# CYTOLETHAL AND GROWTH INHIBITORY EFFECTS OF GLUCOCORTICOIDS ON HUMAN LYMPHOID CELLS

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## DECLARATION

This thesis has been composed entirely by myself.

The work described was undertaken as part of the programme of a research team. The results are almost entirely my own work, and any results or information contributed by other workers are clearly acknowledged.

(Alison M.G. Robertson)

Edinburgh, March, 1979.

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Others who have worked with our group for short periods have also/...

also been of great help. I would like to thank the two recent honours students, Miss Karen Mayne and Mr. John Au, who undertook projects which contributed results helpful to this thesis. I would like to further acknowledge Karen Mayne, who gave me permission to include some of her data and electron micrographs on the methyl-prednisolone-induced growth inhibitory effect on $EB_4$ cells. These are presented in: Part II, Section 2, figs. 41 and 44; Part II, Section 3, fig. 45; Appendix II, table 14. Her work was partially supervised by myself. I would also like to mention Dr. J. Searle of University of Queensland, Australia who provided helpful methodology for the fixation of cells for electron microscopy, and Miss Judy Read of Ottowa, Canada.

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## SUMMARY

Glucocorticoids cause the regression of certain lymphoid tumours, though the precise mechanism by which this is achieved is still not fully understood. Consequently, I studied the action of a glucocorticoid, methylprednisolone (MPS), on human lymphoid cell lines as a possible in vitro model for this effect.

MPS induced both a cytolethal and growth inhibitory response in these cells, which I have defined on a kinetic and morphological basis.

The cytolethal response - measured by the ability of live cells to exclude nigrosine after treatment over 48hr - was dose-dependent, occurring around $10^{-3}$M MPS. Maximal lethal effects occurred only on continuous exposure of the cells to MPS. Morphological changes, as observed by light and electron microscopy, were consistent with apoptosis followed by autolysis, with increased nigrosine uptake correlating closely with onset of autolysis. A low background level of apoptosis and autolysis was also present in control cultures.

The growth inhibitory response - measured by an increase in population doubling time after treatment over several days - was dose-dependent, occurring within the concentration range $10^{-7}$M - $10^{-4}$M MPS. Again, continuous exposure to MPS was necessary for the response to be maintained. Morphological changes resulting from sub-lethal damage were seen in mitochondria by electron microscopy; in addition a small increase in the number of apoptotic and autolytic cells was observed by light microscopy. Cell cycle kinetic analysis revealed/...

revealed that growth inhibition was a stable effect caused by a blockage of cells in $G_1$ (or $G_0$); I was unable to determine whether blocked cells were committed to death or maintained the ability to return to cycle.

A correlation was made between my own results and biochemical studies performed on human lymphoid cell lines by other workers in our research group. The relationship between *in vitro* and *in vivo* responses to glucocorticoids was also discussed.

CONTENTS



PART II/...

# GENERAL INTRODUCTION

The growth rate of tumours and of normal renewal tissue is determined by three kinetic factors: (a) the cell cycle time of proliferating cells, (b) the proportion of cells proliferating (i.e., the growth fraction) and (c) the rate of cell loss by death or removal (Steel, 1967; Lamerton, 1976; Tubiana and Malaise, 1976) (see fig. 1). Of these, cell death has been the least studied and it is with this that the first part of my thesis is concerned.

There are at least two major modes of cell death apparent in both normal and malignant tissues - coagulative necrosis and apoptosis. Their morphological appearance and occurrence differ markedly which suggests mediation by different mechanisms.

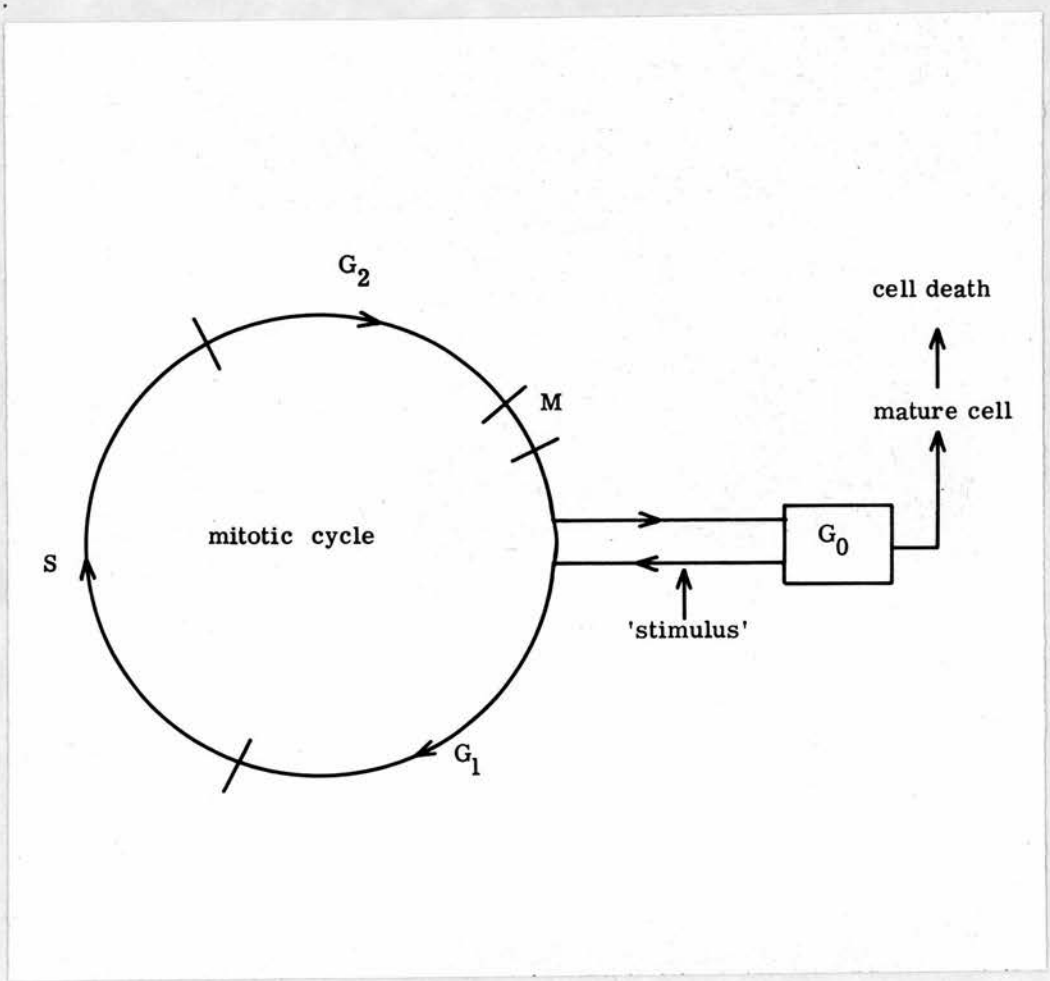
Coagulative necrosis results from the breakdown of normal homeostatic control mechanisms (Trump and Ginn, 1969) which leads to a change in permeability of the plasma membrane and loss of cell volume control. This is measured by loss of normal membrane potential (Saladino and Trump, 1968; Jennings, Ganote and Reimer, 1975) and change in ionic flux resulting in potassium loss and entry of sodium and water into the cell (Laiho and Trump, 1974).

Early morphological changes involve swelling of the cytoplasm and organelles accompanied by patchy nuclear chromatin aggregation and condensation (pyknosis) around the periphery of the nucleus; later stages involve disorganisation and rupture of the plasma membrane/...

Fig. 1

Schematic representation of the cell cycle in tumours and normal renewal tissue

Proliferating cells pass through various phases of the mitotic cycle (Howard and Pelc, 1953): $G_1$, post-mitotic phase of variable duration; S, DNA-synthetic phase; $G_2$, relatively short pre-mitotic phase; M, period of mitotic division. A proportion of cells may be 'resting' in $G_0$ phase (i.e., growth fraction < 100%) where they maintain their capacity to be 'stimulated' back into the division cycle (Lajtha, 1963). Non-proliferating mature cells have a limited life-span and are 'committed' to death.
This scheme excludes the possibility of '$G_2$-arrest' cells known to occur in other tissues (Hill, 1976).



membrane and dissolution of intracellular structures, with fragmentation of chromatin clumps (karyorrhexis) followed by chromatin dissolution (karyolysis) (Trump and Ericsson, 1965; Trump and Arstila, 1971). Coagulative necrosis usually involves large numbers of adjacent cells in tissues with no dissociation of junctional complexes until cell death is well advanced (Trump and Ginn, 1969). There is total disruption of structure of affected areas and there is usually associated inflammation in surrounding viable tissues (Majno, 1964).

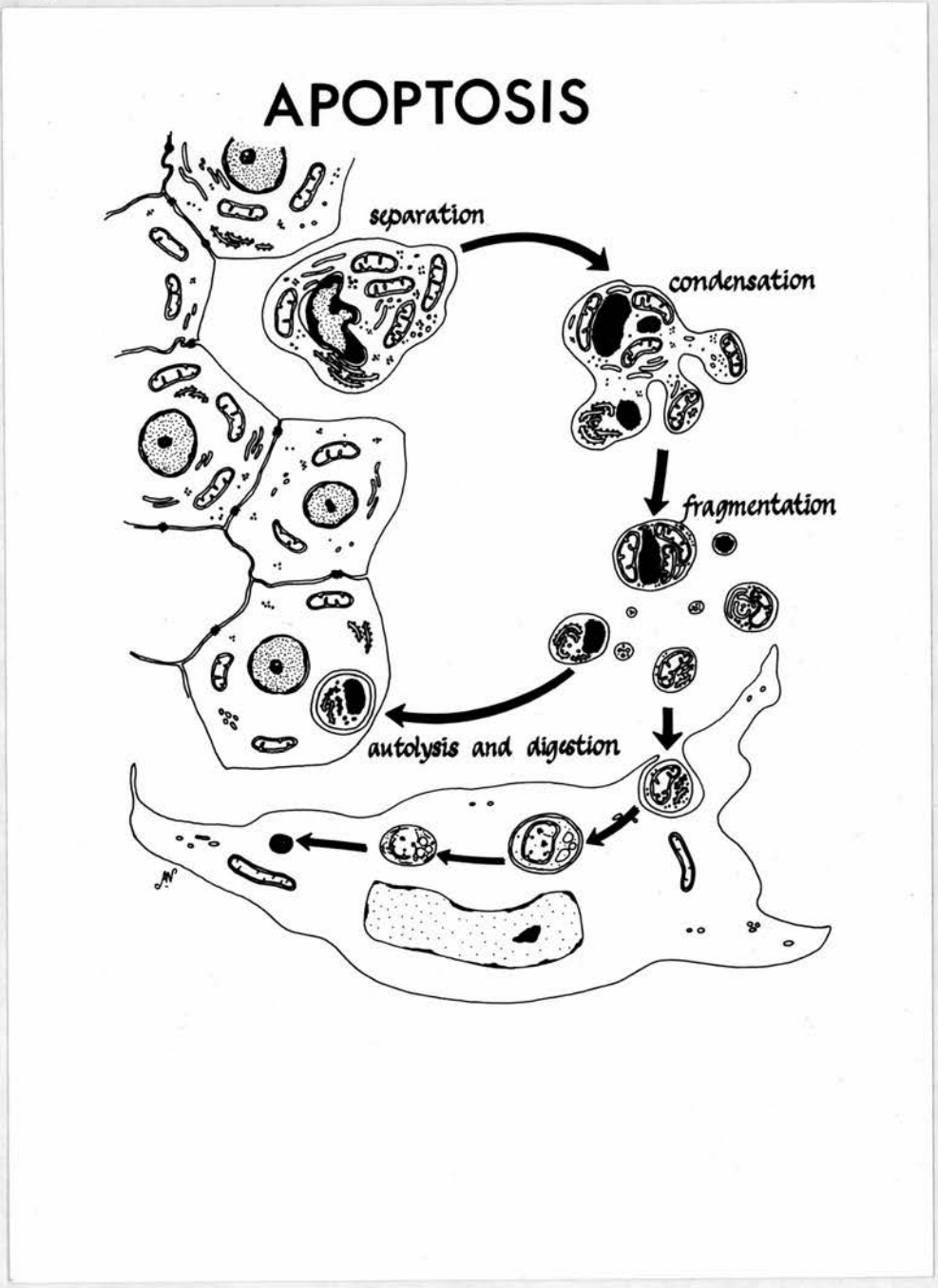
Coagulative necrosis occurs: in states of anoxia e.g., in ischaemic tissue injury (Jennings, Ganote and Reimer, 1975) or in tumours due to vascular insufficiency (Thomlinson and Gray, 1955; Tannock, 1968); due to inhibition of oxidative phosphorylation, glycolysis or citric acid cycle (Saladino and Trump, 1968; Hawkins et al, 1972; McDowell, 1972a, 1972b; Laiho and Trump, 1974); on exposure of plasma membranes to agents such as toxins (McLean, McLean and Judah, 1965) and complement (Hawkins et al, 1972); in in vitro autolysis (Trump, Goldblatt and Stowell, 1965; Trump and Ginn, 1969).

Apoptosis, a morphologically distinctive type of cell death (Kerr, Wyllie and Currie, 1972) affects scattered single cells without surrounding structural damage or inflammation. In some situations it appears to play a rôle complementary to mitosis in the controlled regulation of normal (Wyllie et al, 1973) and tumour cell populations (Kerr, Wyllie and Currie, 1972).

The essential features of apoptosis are shown diagrammatically in fig. 2. Affected cells initially round up losing contact with their/...

Fig. 2

Diagram to illustrate the morphological features of apoptosis (from Kerr, Wyllie and Currie, 1972)



their neighbours by junctional complex breakdown, show condensation and peripheral aggregation of nuclear chromatin and condensation of cytoplasm and organelles. This is followed by nuclear membrane breakdown and nuclear fragmentation, accompanied by 'budding' or 'blebbing' of the cell to form morphologically distinctive membrane-bounded apoptotic bodies which may or may not contain nuclear remnants, and in which cytoplasmic organelles maintain their structural integrity. Later stages involve phagocytosis of these bodies by neighbouring unaffected parenchymal cells or histiocytes, where they undergo destruction within phagosomes by fusion with cellular lysosomes. This results in their progressive degradation, but ultimately undigestible material may remain in the ingesting cell as a lysosomal residual body. Release of lysosomal enzymes is not considered as being involved in the genesis of apoptosis (Kerr, 1972).

Apoptosis appears to be a widespread phenomenon and a detailed presentation of its incidence in vivo can be found in Kerr, Wyllie and Currie (1972) and Wyllie (1974). The typical morphological features of apoptosis have been reported in the following situations: during embryonic morphogenesis in the involution of tissues e.g., in the removal of interdigital webs in rodents (Ballard and Holt, 1968) and reptiles (Fallon and Cameron, 1977); in teratogenesis (Crawford, Kerr and Currie, 1972); during metamorphosis e.g., in epidermal and skeletal muscle cell death in the regression of the anuran tadpole tail (Kerr, Harmon and Searle, 1974); in hormone-induced atrophy e.g., in castration-induced involution of the prostate due to androgen withdrawal (Kerr and Searle, 1973) or in the adult adrenal cortex after withdrawal of adrenocorticotrophin (ACTH) secretion by prednisone administration/...

administration (Wyllie et al, 1973); on exposure of tissues to anoxia or toxins where environmental conditions are not altered sufficiently to cause coagulative necrosis e.g., in ischaemia-induced atrophy of the liver by ligation of the portal blood supply (Kerr, 1971); after exposure of tissues to irradiation e.g., in irradiation of intestinal epithelium and skin epidermis (Potten, Al-Barwari and Searle, 1978).

Of more relevance to the work in this thesis, apoptosis is also present: in normal healthy tissues in the normal physiological control of cell populations (Wyllie, Kerr and Currie, 1973); and in untreated tumours e.g., in basal cell carcinoma of the skin (Kerr and Searle, 1972a, 1972b) and in squamous carcinoma of the uterine cervix (Searle et al, 1973). Apoptosis may be enhanced in therapeutically-induced tumour regression e.g., in irradiation of human squamous cell carcinoma (Kerr and Searle, 1972b) and in mouse ascites tumours after addition of non-steroidal cancer chemotherapeutic agents (Searle et al, 1975). Apoptosis has also been seen in in vitro cell death e.g., in rodent lymphoid cells after treatment with glucocorticoids (Wyllie, in preparation) and after irradiation (Trowell, 1966).

The enhancement of apoptosis in tumour regression indicates the obvious clinical importance of this mode of cell death. However, whilst the occurrence and morphology of apoptosis are now well established, little is known of the intracellular mechanisms by which it is mediated and controlled.

We therefore set out to establish an experimental in vitro system/...

system for the combined study of the sequence of morphological and biochemical changes occurring in apoptosis. Although systems are known for induction of apoptosis *in vivo*, an *in vitro* system would provide:

(a) results which can be analysed freely without the complexities of an *in vivo* environment

(b) predictable onset of apoptosis such that the earliest morphological and biochemical alteration in cells may be studied.

The lethal effects of glucocorticoids on certain rodent lymphoid cells have been known for many years (Dougherty and White, 1945), and as a result they have been employed extensively in the treatment of human lymphoid tumours (DeVita, 1973; Schein *et al*, 1975; Berard *et al*, 1976). Initially glucocorticoids were used as a single agent in the treatment of lymphoid tumours; however remission, when it occurred, was only achieved for a limited period of several months (Ezdinli *et al*, 1969; Henderson, 1969). During the last decade glucocorticoids have been used more successfully as part of chemotherapeutic régimes involving combined drug therapy, especially in the case of childhood acute lymphoblastic leukaemia (ALL) (Berard *et al*, 1976; Mauer, Simone and Pratt, 1977; Simone *et al*, 1978). However most drug régimes are still designed on an empirical basis and the mechanism by which glucocorticoids act is still not fully understood.

There are two lines of evidence which together suggest that glucocorticoids exert a cytolethal effect on human leukaemic cells: (a) the rapid occurrence of a marked lymphocytopaenia (Henderson, 1969; Lampkin, Nagao and Mauer, 1969; Ernst and Killmann, 1970), and (b) the complication/...

complication of hyperuricaemia in a small percentage of leukaemic patients (the result of massive DNA degradation) (Sandberg, Cartwright and Wintrobe, 1956; Wolff et al, 1967) after glucocorticoid therapy. However there is no direct morphological evidence as yet of the mechanism of deletion involved. Nevertheless, as apoptosis is known to play an important rôle in the regression of certain tumours, then the treatment of neoplastic human lymphoid cells in vitro with glucocorticoids might prove a suitable model for induction of apoptosis.

It would be desirable to use freshly isolated human lymphoid cells from patients with leukaemia and lymphoma for experimental purposes, however several difficulties are attached to this approach:

(a) within each class of leukaemia or lymphoma there may be a range of phenotypes (Leventhal and Konior, 1976; Habeshaw, Macaulay and Stuart, 1977) and this difficulty in precise classification can cause problems when data from different patients are correlated

(b) cells tend to be available most readily from patients already exposed to chemotherapeutic agents

(c) there are ethical constraints on obtaining samples from patients who may be extremely ill or undergoing debilitating chemotherapy.

We therefore decided to use human lymphoid cell lines in permanent culture to study the glucocorticoid-induced cytolethal effect; this system is free from the above difficulties and provides a readily obtainable population of reproducible cell type for repeated analysis.

The/...

The human lymphoid cell lines we have used are derived from patients with leukaemias and lymphomas, from lymphoid cells of peripheral blood, lymph glands or lymphoid tumours. They have been established as permanent cell lines in suspension culture either spontaneously or by a process of co-cultivation with lethally irradiated cells containing Epstein-Barr virus (EBV) (Pulvertaft, 1965; Jensen et al, 1967; Steel and Edmond, 1971; Steel, 1972). All the cell lines studied contain the EBV genome and show certain characteristics of B lymphocytes (Nilsson and Pontén, 1975).

In addition to the cytolethal effect, evidence also suggests that glucocorticoids affect the rate of proliferation of human neoplastic lymphoid cells by exerting a 'cell cycle' or 'growth inhibitory' (retardation) effect - this has been shown both in freshly isolated leukaemic blast cells from bone marrow aspirates (Lampkin, Nagao and Mauer, 1969, 1971; Ernst and Killmann, 1970) and in certain cultured lymphoid cell lines from patients with lymphoid neoplasia (Hirshaut, Weiss and Perry, 1969; Nilsson, 1971; Norman and Thompson, 1977). Thus the growth inhibitory effect may also be an important component of the therapeutic activity of glucocorticoids. However again, the intracellular mechanisms by which this affect may be achieved are poorly understood, and hence we have also studied this effect in human lymphoid cell lines, results of which are presented in the second part of my thesis.

As well as their growth inhibitory effect on human neoplastic lymphoid cells, glucocorticoids have also been shown to slow the rate of proliferation in many other cell types both in vivo: in a

spontaneous/...

spontaneous rodent mammary tumour (Braunschweiger, Stragand and Schiffer, 1978); and *in vitro*: in HeLa cells (Kollmorgen, 1969; Adolphe and Lechat, 1974), in inhibition of PHA-stimulation of normal human lymphocytes (Mendelsohn, Multer and Bernheim, 1977), in human lung alveolar cells (Jones, Anderson and Addison, 1978), in connective tissue fibroblasts (Nacht and Garzón, 1974), in rat glioma cells (Grasso *et al*, 1977), in primary human skin and mouse fibroblasts (Ponec *et al*, 1977) and in mouse lymphoma cells (Story and Melnykovych, 1973).

From some of these studies both *in vivo* and *in vitro* it has been reported that the growth inhibitory effect occurs due to an accumulation of cells in the $G_1$ or $G_0$ phase of the cell cycle i.e., a blockage of the transition of $G_1$ cells into the S-phase (Ernst and Killmann, 1970; Lampkin, McWilliams and Mauer, 1972; Mendelsohn, Multer and Bernheim, 1977; Norman and Thompson, 1977; Braunschweiger, Stragand and Schiffer, 1978). *In vitro* synchronised cell populations have also been used to determine the point in the cell cycle at which glucocorticoid effects are manifested and studies with HeLa cells (Adolphe and Lechat, 1974) have shown that the cells are least sensitive to glucocorticoid during the S-phase and most sensitive in $G_1$.

At present, however, there is confusion over the fate of the glucocorticoid-arrested cells as to whether they are then irrevocably committed to die (Norman, Harmon and Thompson, 1978) or whether they can be recruited back into cycle (Kollmorgen, 1969; Braunschweiger, Stragand and Schiffer, 1978); hence this has formed part of our study on/...

on human lymphoid cell lines.

- The morphology of the glucocorticoid-induced growth inhibitory response has also received scant attention and we have therefore studied this in some detail. Sub-lethal injury - which could however be involved in this response - has been described by Werthamer and Amaral (1975) in the treatment of normal human lymphocytes with cortisol. Cytoplasmic organelles were affected, mitochondria in particular, which showed deterioration in the formation of myelin figures denoting an autophagic activity. In addition the golgi became very pronounced in structure, and occasionally there was reorganisation of the endoplasmic reticulum resulting in a bizarre array of parallel cisternae-like structures.

Much of the effort in elucidating the biochemical mechanism of action of glucocorticoids has come from studies on rodent lymphoid cells, in particular on rat thymocytes and steroid-sensitive mouse lymphoma cell lines (see reviews by Claman, 1972; Thompson and Lippman, 1974; Baulieu, 1975; Rosen and Milholland, 1975; Higgins and Gehring, 1978). Rodent lymphoid cells are very sensitive to the effects of glucocorticoids and indeed the cytolethal response is well documented (Dougherty and White, 1945; Burton, Storr and Dunn, 1967; Whitfield, Perris and Youdale, 1968; Harris, 1970; Claman, 1972; Turnell, Clarke and Burton, 1973; Leung and Munck, 1975).

Although at present no proof exists that such a mechanism of action is involved in the lethal response to glucocorticoids in human lymphoid cells, the currently accepted model for steroid hormone/...

hormone action is illustrated in fig. 3. The steroid enters the cell probably by passive diffusion in the case of glucocorticoids where it binds to high affinity specific protein receptor molecules present in the cytoplasm. The hormone-receptor complex then undergoes a temperature-dependent ( $\geqslant 20^{\circ}C$) conformational change (activation) and migrates to the nucleus, binding to nuclear acceptor sites on the chromatin. This influences transcriptional activity of specific parts of the genome and the altered gene products ultimately mediate the steroid effects. The exact means by which the steroid-receptor complex evokes these responses is as yet unknown, but an increased accumulation of functional mRNA for the specific induced protein has been demonstrated (Schutz et al, 1975; Bell and Borthwick, 1976).

From the model, this induced cell response can then lead to secondary inhibition effects; for example in rodent thymocytes, glucocorticoids cause inhibition of glucose uptake and of RNA and DNA synthesis (Makman, Nakagawa and White, 1967), and similar inhibitory responses have been observed in mouse L cells (embryonic fibroblast line) (Pratt and Aronow, 1966). All of these effects are inhibited by actinomycin D and cycloheximide (Mosher, Young and Munck, 1971; Baran, Lichtman and Peck, 1972) and are therefore presumably dependent at some stage on macromolecular synthesis.

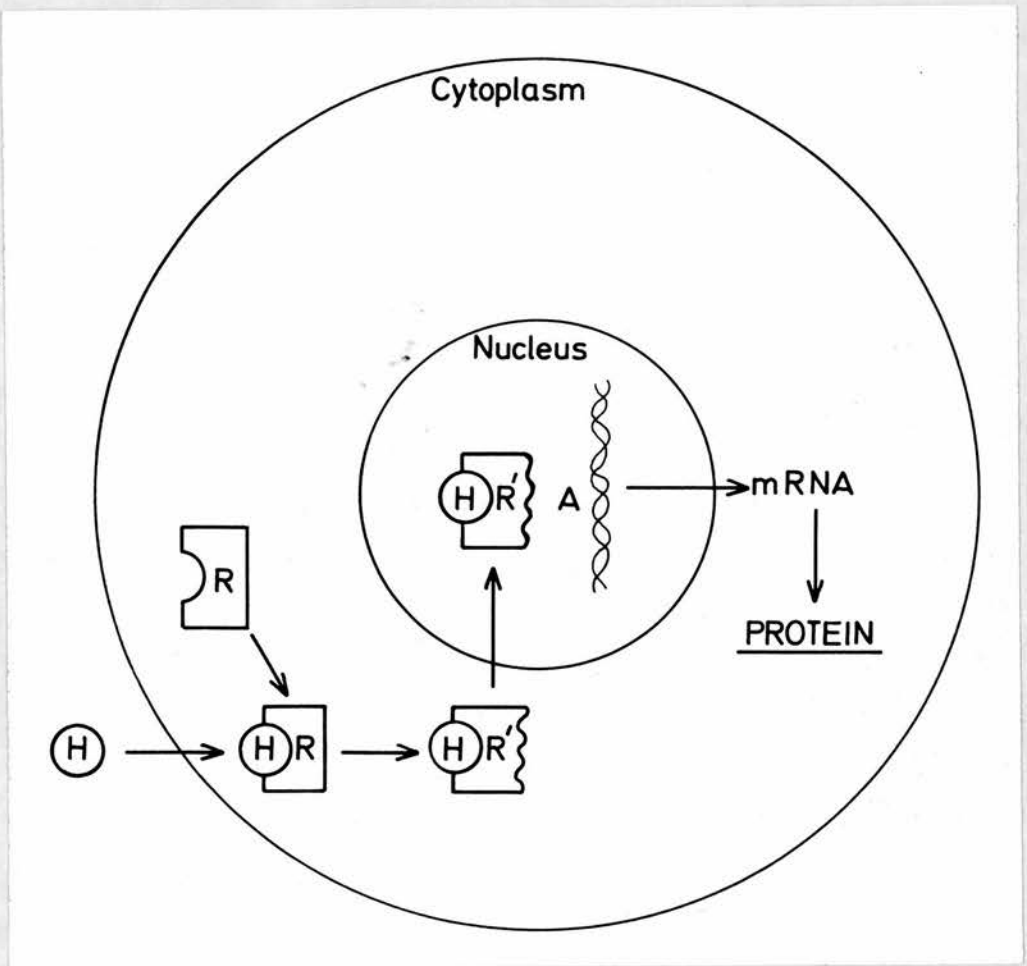
Compared to rodent lymphoid cells, human lymphoid cells appear relatively insensitive to glucocorticoid (Claman, 1972), and hence little work has been directed towards studying the biochemical mechanism of action of glucocorticoids with human material. The rôle of specific cytoplasmic receptors in steroid responsiveness in human leukaemia/...

Fig. 3

Model of the mechanism of steroid hormone action

Abbreviations:

| | | |
|---|---|---|
| H | - | hormone or steroid |
| R | - | receptor |
| R' | - | activated receptor (conformational change) |
| A | - | nuclear acceptor site (chromatin) |
| mRNA | - | processed messenger RNA |



leukaemia cells, however, is being investigated and some results would suggest that the presence of specific glucocorticoid receptors is necessary for the patient to show a therapeutic response (Gailani et al, 1973; Lippman et al, 1973; Lippman, Perry and Thompson, 1975; Yarbro et al, 1977). Thus the model of steroid hormone action could be applied to glucocorticoid leukaemia therapy where the production of altered gene products mediate effects which can induce either cell death or growth inhibition in the leukaemic cell population, thus causing the observed remission - what these gene products are and how the effects are manifested are problems yet to be resolved. It has also still to be resolved what rôle receptors play in acquired resistance of leukaemic patients to glucocorticoid therapy and indeed how useful the glucocorticoid receptor assay in vitro is for determining the clinical response in vivo.

In summary, it is not clear (a) whether glucocorticoid induces remission in human lymphoid tumours by causing cell death and/or growth inhibition, (b) what morphological changes may be involved in the response of human lymphoid tumour cells to glucocorticoid and (c) what is the precise biochemical basis for glucocorticoid sensitivity and resistance in human lymphoid cells.

I have therefore studied the kinetics and morphology of the cytolethal and growth inhibitory responses induced by the glucocorticoid, methylprednisolone, on human lymphoid cell lines and attempted to correlate the results with biochemical investigations performed by other members of the group. Hopefully we may be able to enlarge our basic understanding of how glucocorticoids act at the cellular level in/...

in this test system, and this may result in more rational therapeutic régimes for the treatment of patients with lymphoid tumours.

# PART I CYTOLETHAL GLUCOCORTICOID EFFECTS ON HUMAN LYMPHOID CELLS

## SECTION 1

## KINETICS OF THE CYTOLETHAL RESPONSE OF HUMAN LYMPHOID CELLS TO GLUCOCORTICOID

Initially it was necessary to establish an experimental system to study the concentration of glucocorticoid necessary to produce a cytolethal response in human lymphoid cells.

Growth characteristics of the cell lines were studied and experiments were then performed on cultures in logarithmic growth phase to ensure that 'non-specific' effects such as 'exhaustion' of nutrients in the growth medium did not contribute to the cytolethal effects of the glucocorticoid.

The cytolethal response was tested with glucocorticoid within the concentration range $10^{-7}$M - 2 x $10^{-3}$M. In all experiments, the cytolethal response was assessed at 48hr after treatment with the glucocorticoid by the ability of live cells to exclude the vital dye nigrosine.

The kinetics of the cytolethal response was studied over a 48hr incubation period with the glucocorticoid, and experiments conducted to determine whether the continued presence of glucocorticoid was necessary for a cytolethal response to occur.

As a comparison with our in vitro model, the cytolethal response of/...

of normal human peripheral blood lymphocytes to glucocorticoid was also investigated.

## MATERIALS AND METHODS

### Cell culture

(a) Lymphoid cell lines. These were obtained from the MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh. Their origins are shown in table 1.

TABLE 1 ORIGIN OF HUMAN LYMPHOID CELL LINES

| Cell line | Origin |
|---|---|
| $BLA_1$ | Acute lymphoblastic leukaemia (ALL) |
| $RUS_2$ | Acute myeloblastic leukaemia (AML) |
| $GS_1$ | Chronic lymphatic leukaemia (CLL) |
| JIJOYE | Burkitt's lymphoma |
| $EB_4$ | Burkitt's lymphoma |
| RAJI | Burkitt's lymphoma |

The cells were grown in suspension culture in conical glass flasks or roller culture bottles in Eagle's minimum essential medium (MEM, GIBCO-Biocult) containing sodium bicarbonate buffer (20mM), glutamine (4mM), streptomycin (80µg/ml) and penicillin (80iu/ml), and supplemented with 20% heat-inactivated (56°C for 1hr) foetal bovine serum (FBS, GIBCO-Biocult). (Heat-inactivation destroys/...

destroys any glucocorticoid-binding capacity of globulins present in the serum.) Cultures were incubated at 37°C and flasks were kept in a humidified atmosphere of 5% $CO_2$ in air. Cells were maintained at densities between 2-10 x $10^5$ cells/ml by feeding with fresh growth medium every 3 - 4 days. Cultures were routinely tested for mycoplasma infection.

(b) Normal peripheral blood lymphocytes. Lymphocytes were obtained from buffycoat 'pig-tail' blood samples from the Blood Transfusion Service, Royal Infirmary of Edinburgh.

Growth medium for lymphocytes was the same as for lymphoid cell lines, but supplemented with 10% heat-inactivated calf serum (CS, GIBCO-Biocult).

The blood was defibrinated by placing in a glass universal container, containing glass beads and growth medium with 1% topical thrombin, followed by agitation on a blood mixer for 10 min. The defibrinated supernatant was removed and layered onto a 9% Ficoll/ 33.9% Triosil mixture in a universal container. This was centrifuged at 1500 x g for 15 min. during which the red blood cells pelleted at the bottom of the universal container and the lymphocytes formed a layer at the interface between the Ficoll/Triosil and the blood/medium supernatant. The lymphocyte layer was removed with a pipette, washed once in growth medium, set up in test-tubes at 5 x $10^5$ cells/ml and incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

Cell/...

Cell counts and viability

The total cell concentration (TCC) was enumerated either using a haemocytometer (Improved Neubauer type) or a Coulter Counter (Model ZF) for which samples were diluted 1 in 50 in Isoton (Isoton II, Coulter Electronics Ltd.).

The cell viability was assessed by the ability of live cells to exclude the vital dye, nigrosine - the dead cells take up nigrosine and are stained black. The cells were mixed with an equal volume of 0.5% (w/v) nigrosine in phosphate-buffered saline and counted in a haemocytometer. The viable fraction of the cells was thus defined as the ratio of cells excluding nigrosine to the total number of cells counted.

From these counts the viable cell concentration (VCC) could be estimated: VCC = TCC x viable fraction.

Growth characteristics of cell lines

Stock cells were centrifuged at 400 x g for 5 min. and the cell pellet resuspended in fresh growth medium. Flasks or test-tubes of cells were then set up in duplicate at a concentration of 1-2 x $10^5$ cells/ml. The viability and TCC were assessed every 24hr until the plateau phase of growth was approached.

Glucocorticoid

Methylprednisolone sodium succinate (MPS, Solumedrone, Upjohn, Ltd.) was dissolved in sterile distilled water at 100 x the required test concentrations.

Cytolethal/...

Cytolethal tests

Stock cells were centrifuged at 400 x g for 5 min. and the cell pellet resuspended in fresh growth medium. Nine flasks or test-tubes of cells were then set up in duplicate at a concentration of $2 \times 10^5$ cells/ml. After 24hr, to allow cells to settle into log growth phase, the duplicate cultures were treated with 1% (v/v) of stock MPS solution to give concentrations within the range $10^{-7}$M - $2 \times 10^{-3}$M. The viability and TCC were assessed at 48hr after treatment.

Five test-tubes of normal peripheral blood lymphocytes were set up in duplicate at a concentration of $5 \times 10^5$ cells/ml (as described on p. 19). Duplicate cultures were treated with 1% (v/v) of stock MPS solution to give concentrations within the range $10^{-7}$M - $10^{-3}$M and the viability was assessed at 48hr after treatment.

In all experiments, control flasks or test-tubes were set up in duplicate and treated with 1% (v/v) of sterile distilled water and assessed at 48hr after treatment as for test cultures.

Kinetics of cytolethal response in cell lines

Three flasks of cells resuspended in fresh growth medium were set up in duplicate at a concentration of $2 \times 10^5$ cells/ml. After 24hr, the duplicate cultures were treated with 1% (v/v) of stock MPS solution to give lethal concentrations of $0.75 \times 10^{-3}$, $1 \times 10^{-3}$ and $2 \times 10^{-3}$M MPS. Duplicate control cultures were treated with 1% (v/v) of sterile distilled water. Samples were removed at selected time intervals throughout the experiment for assessment of viability and TCC.

Duration/...

Duration of exposure to cytolethal concentration of MPS

Nine flasks of cells resuspended in fresh growth medium were set up in duplicate at 2 x $10^5$ cells/ml and treated with 1% (v/v) of stock MPS solution to give a final concentration of $10^{-3}$M MPS. Duplicate cultures were washed three times (at 37°C) with fresh growth medium after the desired period of exposure to MPS and the cell pellet resuspended in the original volume of fresh growth medium which contained no MPS. The viability was assessed before washing and at intervals afterwards up to 48hr.

Nine control flasks of cells were set up in duplicate and treated with 1% (v/v) of sterile distilled water. Control cultures were washed and assessed as for test cultures.

## RESULTS

Growth characteristics of lymphoid cell lines

Growth characteristics were studied over a period of 120-144hr in culture during which time cultures were 'starved' of fresh growth medium.

The log growth curves of the individual cell lines are shown in figs. 4 and 5. An initial short lag phase of growth is seen in most cell lines due to handling of the cells in setting up experiments. This is followed by a log phase of growth which is maintained for about 72hr, until plateau phase or 'stationary' phase of growth is approached - this presumably results from 'exhaustion'/...

Fig. 4

Log growth curves of lymphoid cell lines

Cultures were assessed for total cell concentration (TCC) every 24hr after setting up. ○——○, $BLA_1$; ●——●, $EB_4$; ▲——▲, $GS_1$.

Each point represents the mean ± 1S.E. of 2 or 3 separate experiments each with duplicate observations (error bars are omitted where overlapping occurs).

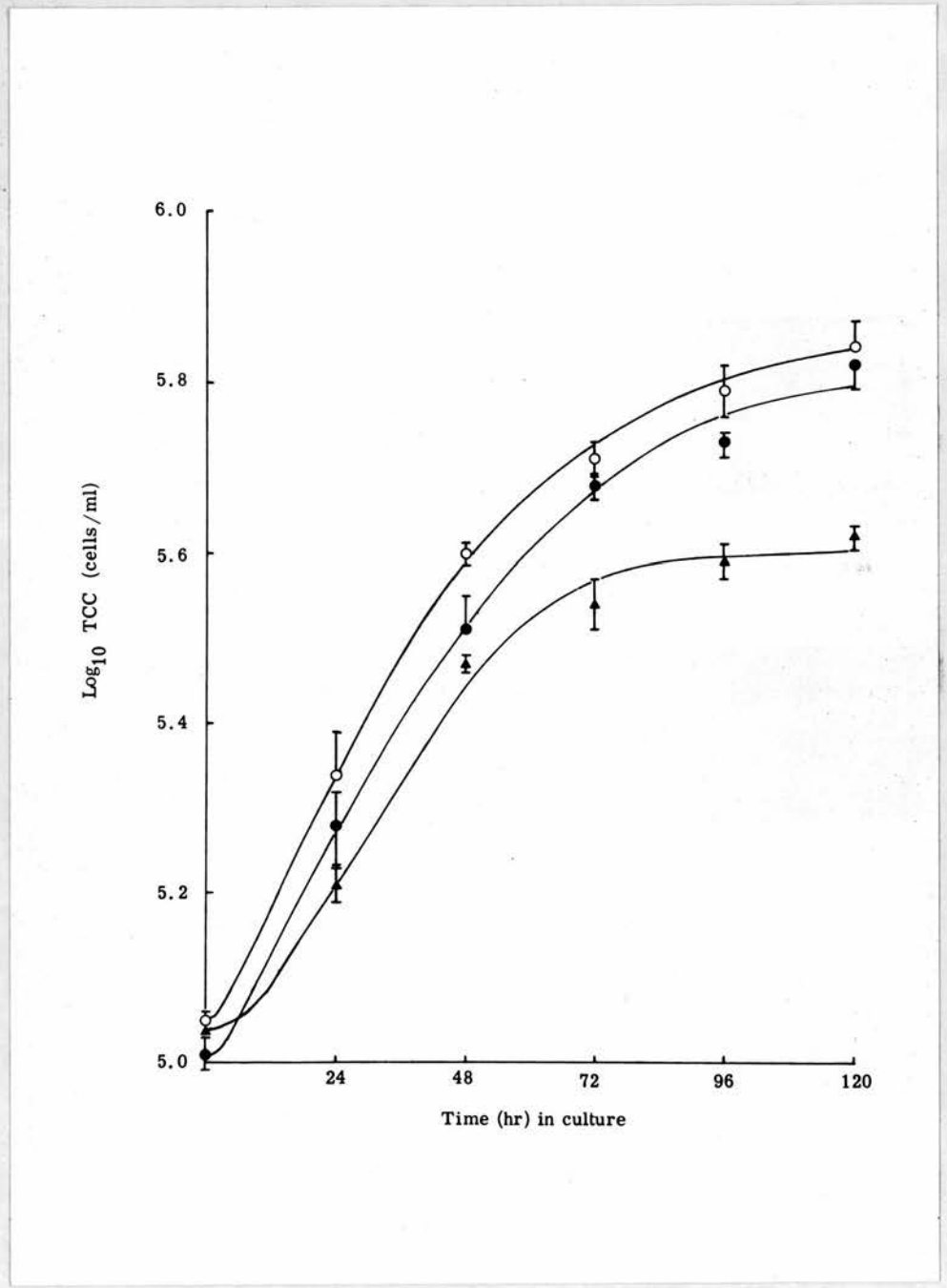
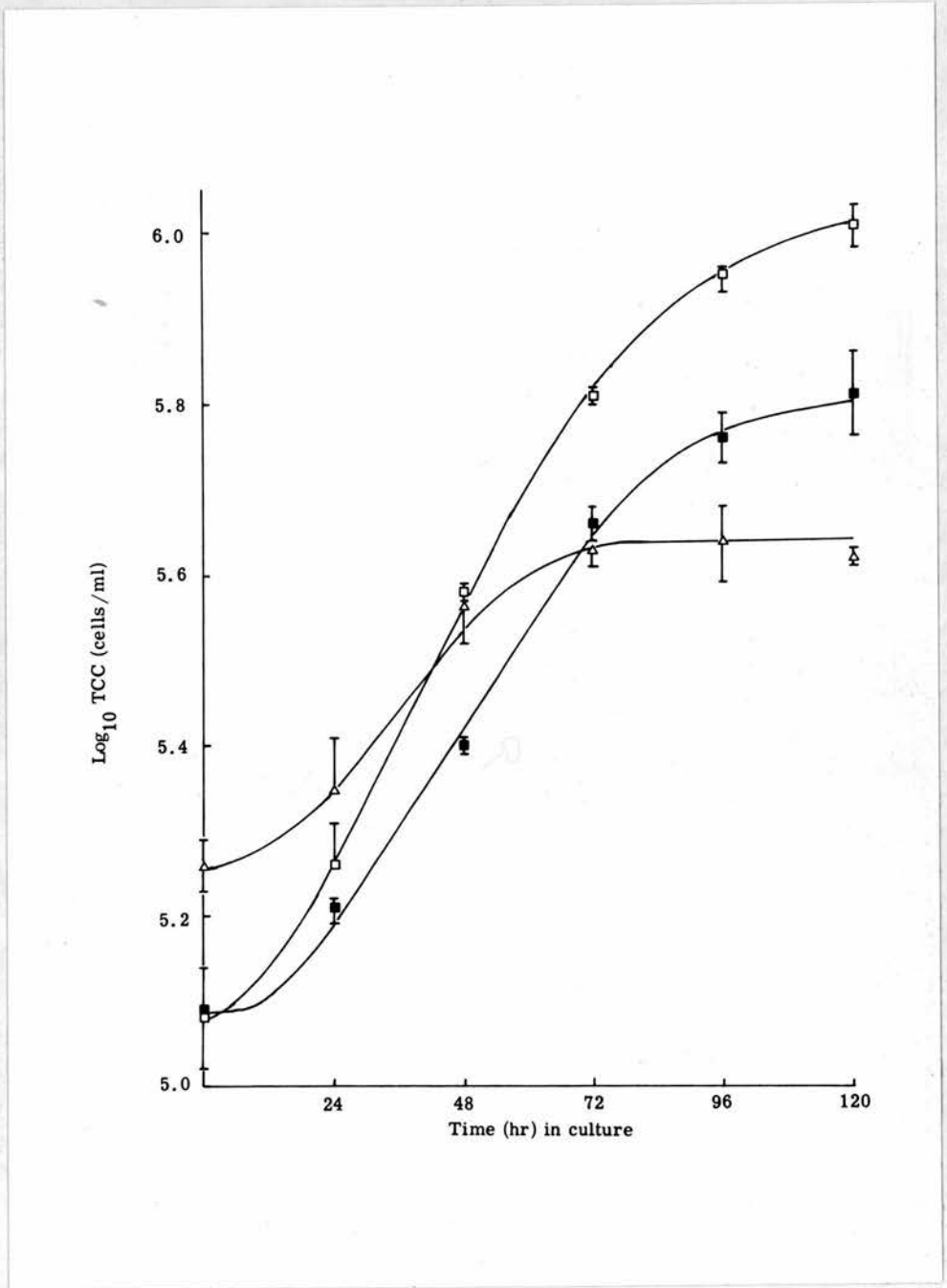


Fig. 5

Log growth curves of lymphoid cell lines

Cultures were assessed for total cell concentration (TCC) every 24hr after setting up. □ —— □, RAJI; ■ —— ■, JIJOYE; Δ —— Δ, $RUS_2$.

Each point represents the mean ± 1S.E. of 2 or 3 separate experiments each with duplicate observations (error bars are omitted where overlapping occurs).



'exhaustion' of nutrients in the growth medium.

The effect of prolonged culture on cell viability of individual cell lines is shown in table 2 (see p. 26). The viable cell fraction remains constant throughout the experiment until plateau phase of growth is approached (see figs. 4 and 5) when it begins to fall.

All the following cytolethal tests were therefore performed on cell cultures which had been set up 24hr previously to ensure log phase of growth and then assessed at 48hr after treatment (72hr after setting up) when no viability change and minimal or no fall in growth rate was observed.

Glucocorticoid sensitivity of lymphoid cell lines

Cultures were treated with MPS concentrations within the range $10^{-7}$M - 2 x $10^{-3}$M. The log dose-response curves of individual cell lines as assessed by viability change after 48hr treatment with MPS are shown in figs. 6-8. All show a typical S-shaped response curve with lethal responses occurring within the range 0.5 x $10^{-3}$ - 2 x $10^{-3}$M MPS. Calculations of $LD_{50}$ values (the median lethal dose) for each cell line as plotted from the graphs are shown in table 3 (see p. 30).

TABLE 3/...

TABLE 2

GROWTH CHARACTERISTICS OF LYMPHOID CELL LINES

| Time (hr) in culture | Viable fraction ± 1S.E. for individual cell lines: | | | | | |
|---|---|---|---|---|---|---|
| | $BLA_1$ | $EB_4$ | $GS_1$ | RAJI | JIJOYE | $RUS_2$ |
| 0 | 0.89 ± 0.02 | 0.90 ± 0.01 | 0.90 ± 0.02 | 0.95 ± 0.02 | 0.83 ± 0.01 | 0.77 ± 0.05 |
| 24 | 0.89 ± 0.02 | 0.91 ± 0.02 | 0.88 ± 0.01 | 0.93 ± 0.01 | 0.77 ± 0.02 | 0.77 ± 0.02 |
| 48 | 0.90 ± 0.01 | 0.91 ± 0.01 | 0.90 ± 0.01 | 0.93 ± 0.01 | 0.78 ± 0.02 | 0.80 ± 0.04 |
| 72 | 0.90 ± 0.01 | 0.90 ± 0.01 | 0.88 ± 0.01 | 0.93 ± 0.02 | 0.85 ± 0.02 | 0.79 ± 0.01 |
| 96 | 0.88 ± 0.01 | 0.88 ± 0.01 | 0.79 ± 0.02 | 0.92 ± 0.02 | 0.85 ± 0.02 | 0.78 ± 0.02 |
| 120 | 0.86 ± 0.02 | 0.88 ± 0.01 | 0.75 ± 0.03 | 0.79 ± 0.05 | 0.76 ± 0.03 | 0.72 ± 0.01 |
| 144 | 0.85 ± 0.02 | 0.75 ± 0.02 | ----- | ----- | 0.55 ± 0.02 | ----- |

The viability of cell cultures was assessed every 24hr after setting up.

Each value represents the mean ± 1S.E. of 2 or 3 separate experiments each with duplicate observations.

-----, no values recorded.

Fig. 6

Effect of methylprednisolone (MPS) on cell viability of lymphoid cell lines ($BLA_1$, $EB_4$)

Cultures were incubated with various concentrations of MPS ($10^{-7}$M - 2 x $10^{-3}$M) (test) or with water (control) and assessed for viability after treatment for 48hr.

O——O, $BLA_1$; ●——●, $EB_4$; - - - -, $LD_{50}$ values (median lethal dose). $LD_{50}$ values are presented in table 3 (see p. 30). Each point represents the mean ± 1S.E. of 3 separate experiments each with duplicate observations. Error bars are omitted where overlapping occurs.

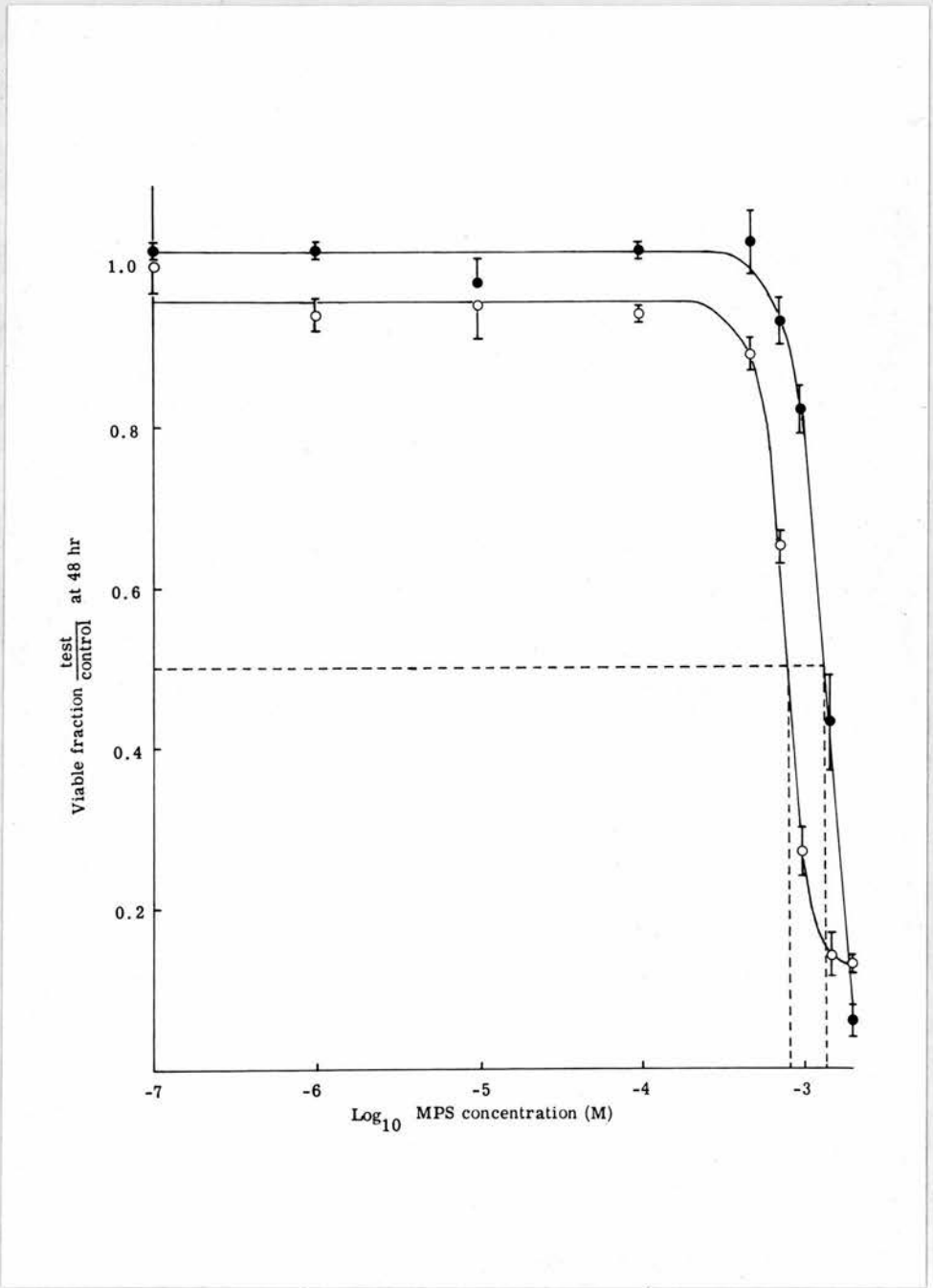


Fig. 7

Effect of methylprednisolone (MPS) on cell viability of lymphoid cell lines ($GS_1$, RAJI)

Cultures were incubated with various concentrations of MPS ($10^{-7}$M - 2 x $10^{-3}$M) (test) or with water (control) and assessed for viability after treatment for 48hr. ▲——▲, $GS_1$; □——□, RAJI; - - - -, $LD_{50}$ values (median lethal dose). $LD_{50}$ values are presented in table 3 (see p. 30). Each point represents the mean ± 1S.E. of 3 separate experiments each with duplicate observations. Error bars are omitted where overlapping occurs.

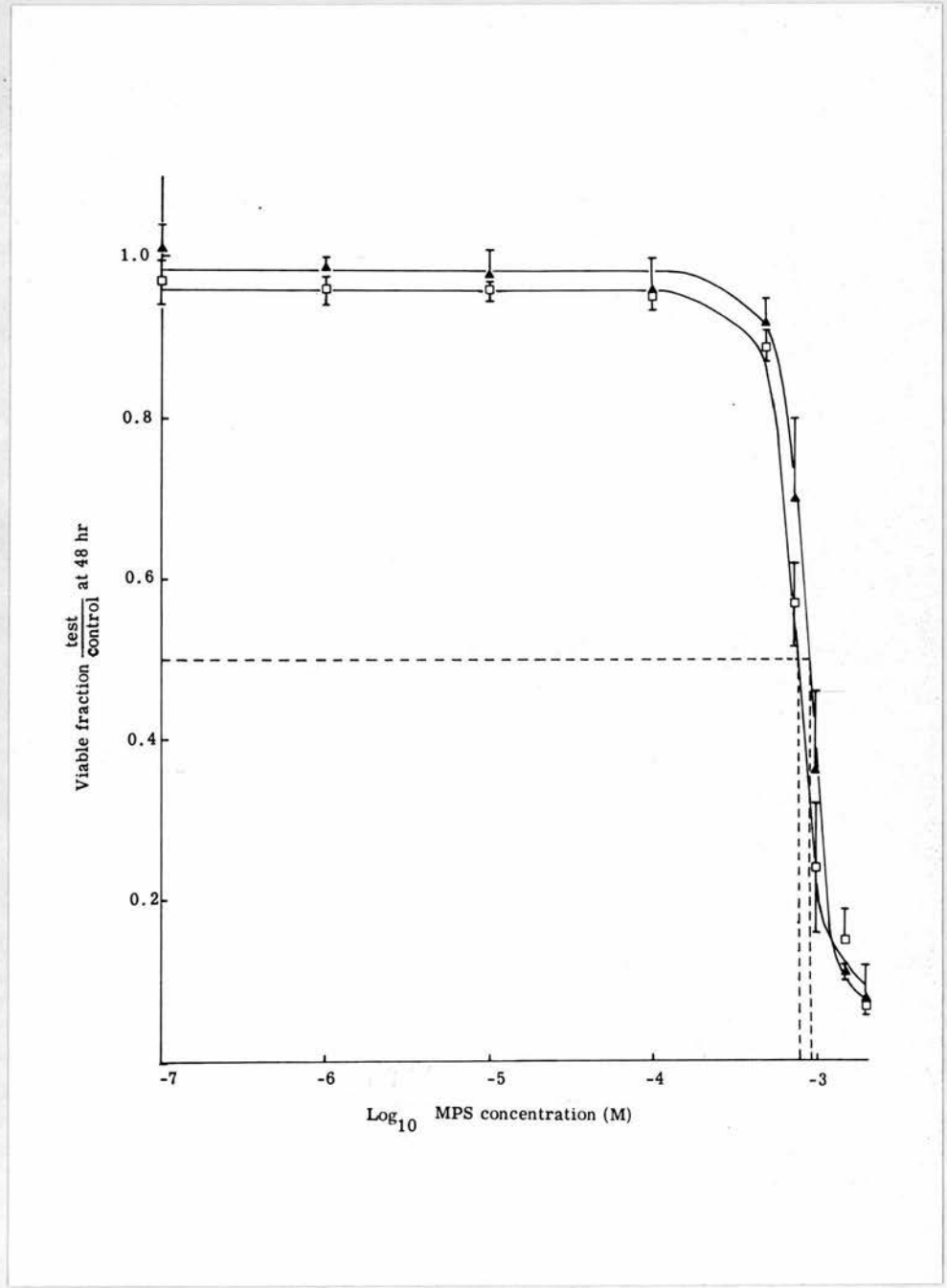


Fig. 8

Effect of methylprednisolone (MPS) on cell viability of lymphoid cell lines (JIJOYE, $RUS_2$)

Cultures were incubated with various concentrations of MPS ($10^{-7}$M - 2 x $10^{-3}$M) (test) or with water (control) and assessed for viability after treatment for 48hr.

■ ——■, JIJOYE; Δ——Δ, $RUS_2$; - - - -, $LD_{50}$ values (median lethal dose). $LD_{50}$ values are presented in table 3 (see p. 30). Each point represents the mean ± 1S.E. of 3 separate experiments each with duplicate observations. Error bars are omitted where overlapping occurs.

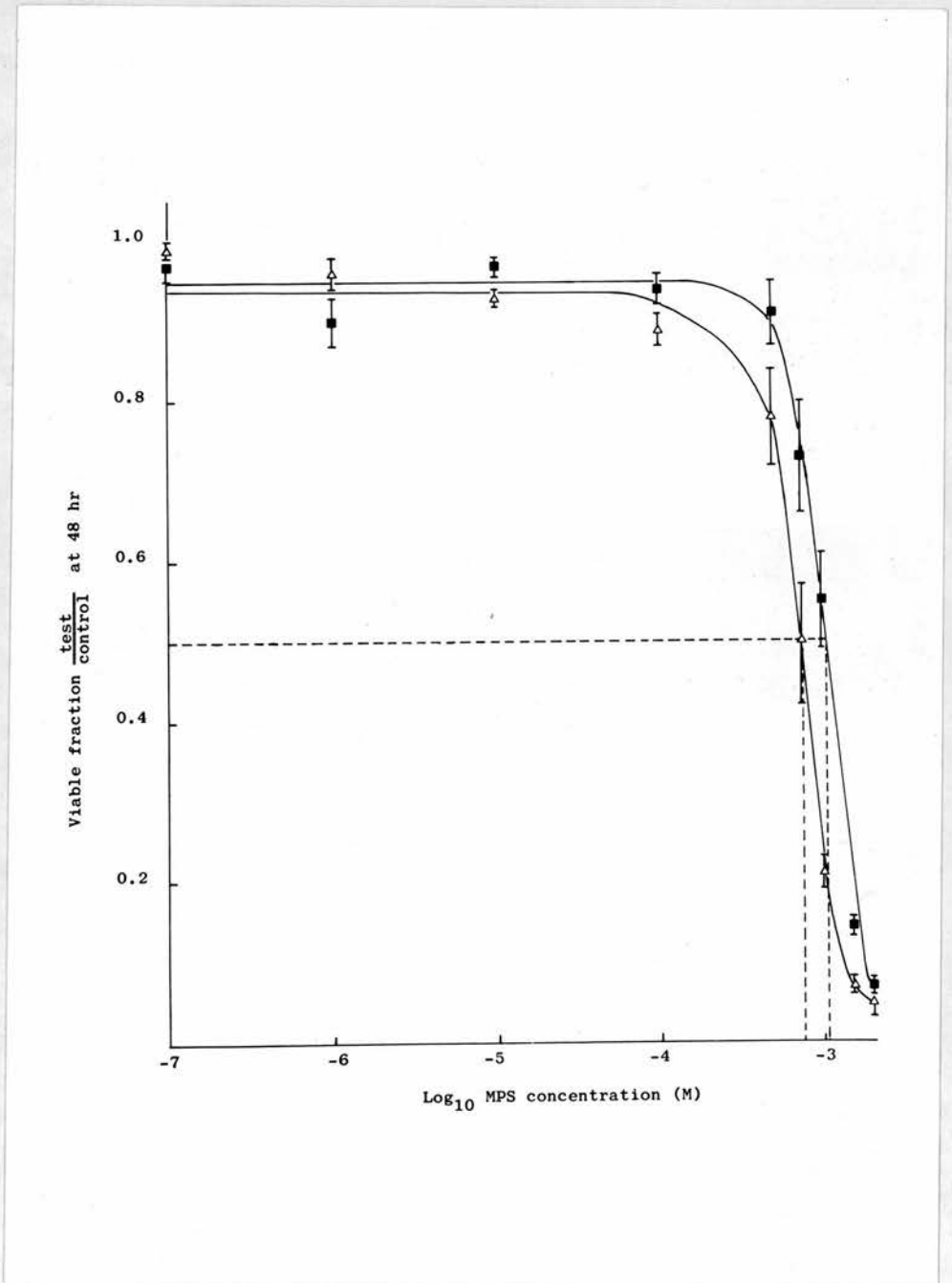


TABLE 3 $LD_{50}$ VALUES FOR LYMPHOID CELL LINES TREATED WITH METHYLPREDNISOLONE (MPS) FOR 48HR

| Cell line | $LD_{50}$* x $10^{-3}$M |
|---|---|
| $BLA_1$ | 0.84 |
| $EB_4$ | 1.14 |
| $GS_1$ | 0.94 |
| RAJI | 0.79 |
| JIJOYE | 1.06 |
| $RUS_2$ | 0.79 |

*the median lethal dose i.e., the concentration of MPS at which the viability of the treated cell population is 50% of the control population viability at 48hr after treatment. Values are obtained from figs. 6-8 (pp.27 -29) as illustrated.

Assessment of TCC at 48hr after treatment showed that the growth rate of cell lines also decreased at the same concentrations ($0.5 \times 10^{-3}$M - $2 \times 10^{-3}$M) of MPS which caused a decrease in cell viability (table 4, see p. 31). As this decrease in TCC after treatment with MPS was consistently shown in all other cytolethal experiments where measured, and as this change was not considered to be a vital measure of the cytolethal response, then TCC results are not presented further in Part I of this thesis.

Glucocorticoid sensitivity of normal human peripheral blood lymphocytes

Cultures of normal human lymphocytes were treated with MPS concentrations within/...

TABLE 4

EFFECT OF METHYLPREDNISOLONE (MPS) ON CELL GROWTH RATE OF LYMPHOID CELL LINES

| MPS concentration (M) | Total cell concentration $\frac{\text{test}}{\text{control}}$ ± 1S.E. at 48hr | | | | | |
|---|---|---|---|---|---|---|
| | $BLA_1$ | $EB_4$ | $GS_1$ | RAJI | JIJOYE | $RUS_2$ |
| $10^{-7}$ | 0.99 ± 0.10 | 1.14 ± 0.06 | 1.11 ± 0.07 | 0.97 ± 0.05 | 0.96 ± 0.05 | 1.07 ± 0.03 |
| $10^{-6}$ | 0.86 ± 0.02 | 0.93 ± 0.02 | 1.13 ± 0.04 | 1.04 ± 0.06 | 0.99 ± 0.06 | 0.95 ± 0.05 |
| $10^{-5}$ | 0.93 ± 0.05 | 0.81 ± 0.05 | 1.04 ± 0.04 | 1.01 ± 0.09 | 0.95 ± 0.06 | 0.94 ± 0.04 |
| $10^{-4}$ | 0.95 ± 0.07 | 0.86 ± 0.04 | 1.03 ± 0.05 | 0.97 ± 0.03 | 0.93 ± 0.04 | 0.88 ± 0.03 |
| $0.5 \times 10^{-3}$ | 0.85 ± 0.10 | 0.76 ± 0.07 | 0.96 ± 0.07 | 0.84 ± 0.13 | 0.84 ± 0.03 | 0.74 ± 0.05 |
| $0.75 \times 10^{-3}$ | 0.72 ± 0.09 | 0.68 ± 0.03 | 0.87 ± 0.08 | 0.67 ± 0.08 | 0.79 ± 0.04 | 0.63 ± 0.04 |
| $10^{-3}$ | 0.63 ± 0.08 | 0.61 ± 0.02 | 0.90 ± 0.09 | 0.58 ± 0.07 | 0.68 ± 0.04 | 0.53 ± 0.06 |
| $1.5 \times 10^{-3}$ | 0.59 ± 0.08 | 0.57 ± 0.03 | 0.82 ± 0.08 | 0.50 ± 0.05 | 0.55 ± 0.04 | 0.48 ± 0.07 |
| $2 \times 10^{-3}$ | 0.58 ± 0.04 | 0.59 ± 0.03 | 0.79 ± 0.08 | 0.52 ± 0.06 | 0.53 ± 0.03 | 0.47 ± 0.07 |

Cultures were incubated with various concentrations of MPS (test) or with water (control) and assessed for total cell concentration after treatment for 48hr.

Each value represents the mean ± 1S.E. of 3 separate experiments.

within the range $10^{-7}$M - $10^{-3}$M. The dose-response curve as assessed by viability change after 48hr treatment with MPS is shown in fig. 9.

A small lethal response was observed only with $10^{-3}$M MPS.

Kinetics of cytolethal glucocorticoid response

The kinetics of the cytolethal response of cells to MPS was studied at concentrations within the range 0.75 x $10^{-3}$M - 2 x $10^{-3}$M, known to produce a lethal effect by 48hr after treatment.

The effect on cell viability of $BLA_1$ cells is shown in fig. 10. This also represents the typical kinetic curves obtained for the other cell lines studied-RAJI, JIJOYE, $GS_1$ and $EB_4$. With the higher concentrations (1 x $10^{-3}$M and 2 x $10^{-3}$M) a brief lag phase was observed before progressive lethal effects ensued, whilst with the lower concentration (0.75 x $10^{-3}$M) there was a more protracted latent interval.

Duration of glucocorticoid pulse and cytolethal response

The duration of the glucocorticoid pulse to which $RUS_2$ cells were exposed was varied by washing cultures free of a lethal dose of MPS ($10^{-3}$M) at intervals up to 48hr.

Fig. 11 shows the effect of washing cultures free of MPS on cell viability. It was found that continuous exposure of cells to MPS was necessary for maximal progression of lethal effects.

COMMENT/...

Fig. 9

Effects of methylprednisolone (MPS) on viability of normal human peripheral blood lymphocytes

Cultures were incubated with various concentrations of MPS ($10^{-7}$M - $10^{-3}$M) (test) or with water (control) and assessed for viability after treatment for 48hr.

Each point represents the mean of duplicate observations from one experiment.

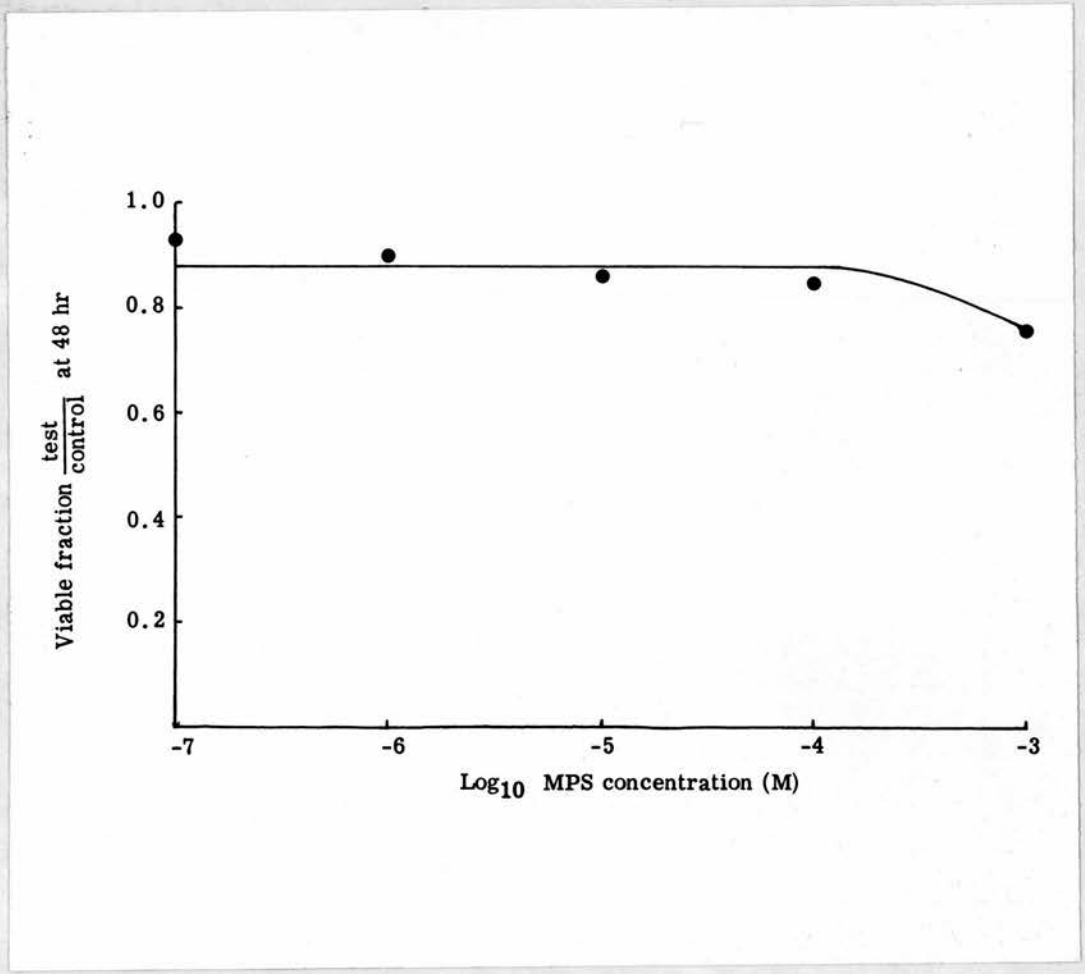


Fig. 10

Kinetics of methylprednisolone (MPS) lethal response in $BLA_1$ cells

Cultures were incubated with water (control) or with various concentrations of MPS (test): O——O, $0.75 \times 10^{-3}$M; Δ——Δ, $1 \times 10^{-3}$M; ●——●, $2 \times 10^{-3}$M. Samples were removed at the times shown up to 48hr after treatment, and assessed for viability.

Each point represents the mean ± 1S.E. of 3 separate experiments each with duplicate observations. Error bars are omitted where overlapping occurs.

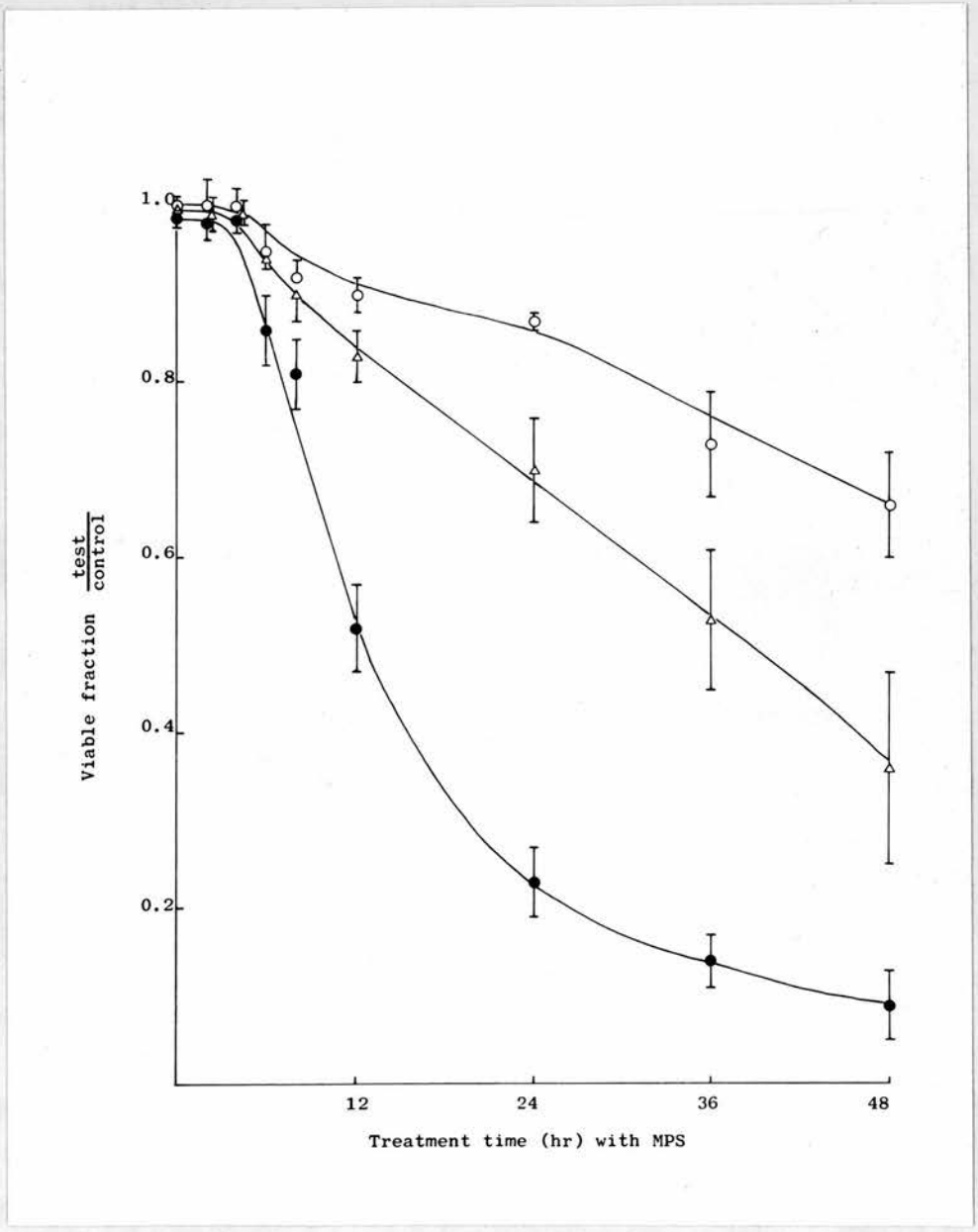
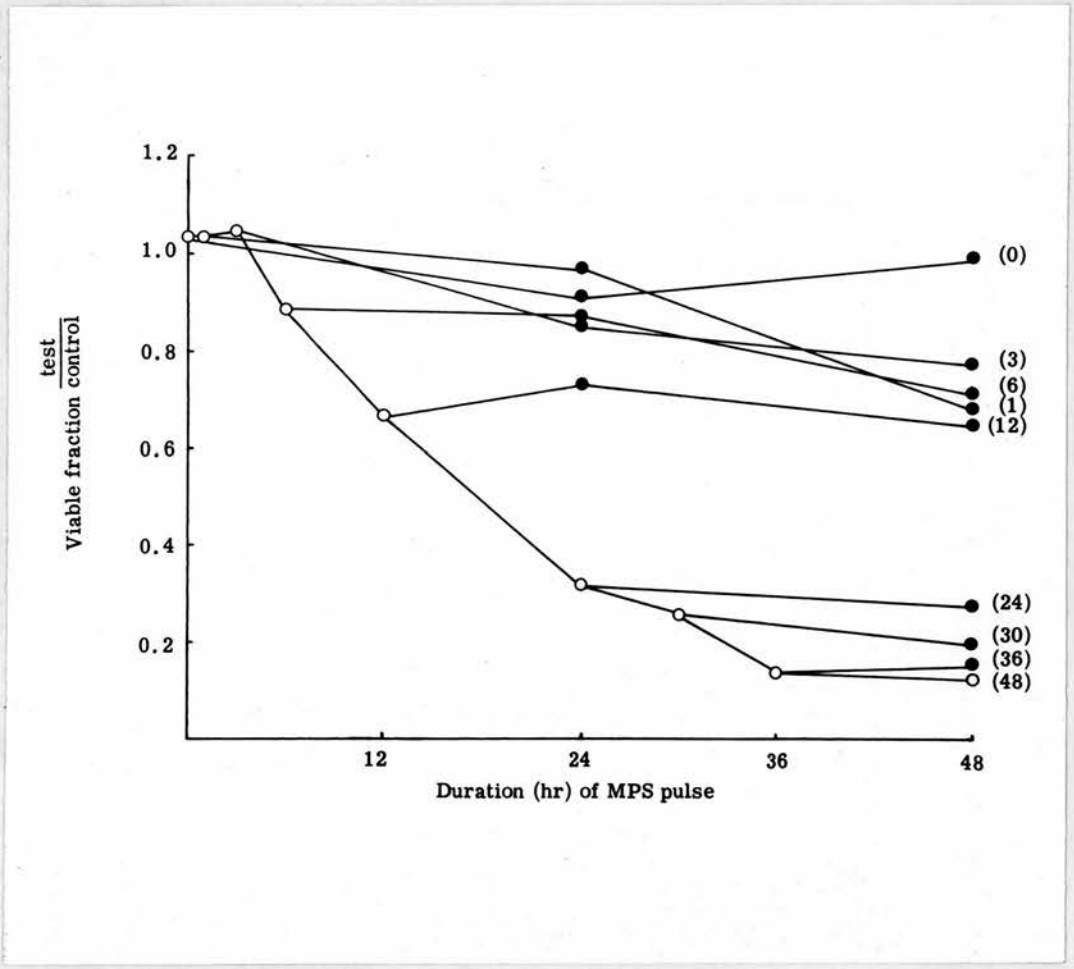


Fig. 11

Duration of methylprednisolone (MPS) ($10^{-3}$M) pulse and cytolethal effect on $RUS_2$ cells

Cultures were incubated with MPS (test) or water (control) for various periods of time up to 48hr. Treatment was removed by washing cells at the times (hr) shown (parenthesis). Cell viability was assessed before washing (O——O) and at intervals afterwards (●——●) up to 48hr.
Each point represents the mean of 2 or 3 separate experiments each with duplicate observations.



COMMENT

The cytolethal experiments on lymphoid cell lines were carried out on cultures in logarithmic growth phase to ensure that cells were in a 'healthy' metabolic state and that adverse culture conditions were not responsible for any decrease in viability.

All cell lines are sensitive to lethal effects of MPS in the region of $10^{-3}$M as shown by dose-response curves and $LD_{50}$ values which range from 0.79 - 1.14 x $10^{-3}$M at 48hr after treatment. The kinetic studies of the lethal glucocorticoid response are consistent with a dose-dependent relationship, with maximal lethal effects occurring only where there is continuous exposure of cells to the glucocorticoid.

However, the relationship between the concentration of glucocorticoid required to induce this cytolethal effect in vitro and that which induces remission induction in vivo is uncertain. It seems that lethal effects in vitro can only be achieved with doses of glucocorticoid which greatly exceed pharmacological levels in vivo. If it is assumed that equilibration of steroids within fluid compartments is equal and non-concentrative, the highest pharmacological doses of glucocorticoids employed in clinical practice (100 - 500mg/$m^2$ body surface) would achieve peak intracellular concentrations around 1-5 x $10^{-5}$M. Maximum physiological plasma steroid levels are generally considered to be about $10^{-6}$ - $10^{-7}$M. much of which is probably bound to plasma corticosteroid binding globulin/...

globulin (transcortin) (Burton and Westphal, 1972).

There is no observed difference in response to MPS between cell lines derived from normally glucocorticoid sensitive diseases such as ALL (Mauer, Simone and Pratt, 1977) or more glucocorticoid resistant diseases such as Burkitt's lymphoma and AML (Brearley et al, 1977; Mauer, Simone and Pratt, 1977), and indeed normal human peripheral blood lymphocytes show the same resistance to pharmacological dose levels. In another study in this laboratory, lymphoid cells freshly derived from the peripheral blood of 6 patients with ALL also showed similar resistance prior to treatment (Bird et al, 1975), despite the patients later undergoing an apparent remission to chemotherapy which included prednisolone.

This observed insensitivity of cultured lymphoid cells cannot be attributed to experimental design, as optimum conditions for the cytolethal response were established by us (Bird et al, 1977) and were employed throughout my experiments - these include studies on (a) glucocorticoid type, (b) glucocorticoid solvent, (c) density of cell culture and (d) heat-inactivation of serum.

Human lymphoid cells in vitro thus appear to exhibit resistance to the lethal effect of glucocorticoid at concentrations which are normally effective in in vivo therapy. In this, they differ markedly from rodent lymphoid cells in vitro which are much more sensitive to physiological doses of glucocorticoid (Claman, 1972).

The correlation between cytoplasmic glucocorticoid receptor levels/...

levels in human lymphoid cells *in vitro* and their cytolethal response to MPS is discussed later (see General Discussion, p. 155).

SECTION 2

## MORPHOLOGY OF THE CYTOLETHAL RESPONSE OF HUMAN LYMPHOID CELLS TO GLUCOCORTICOID

The morphological changes induced by lethal concentrations of glucocorticoids on lymphoid cells *in vitro* have been studied mainly using rodent tissues and conflicting observations have been reported.

Burton, Storr and Dunn (1967) and Whitfield, Perris and Youdale (1968) working with glucocorticoid-induced cell death in mouse and rat thymocytes and the steroid-sensitive mouse lymphosarcoma P1798 sub-line, reported ultrastructural changes which focus on severe nuclear change - the nucleus loses its normal chromatin pattern becoming swollen and homogeneous in structure to give a 'pyknotic' or 'structureless' appearance. The nuclear and cytoplasmic membranes then disrupt to give an overall classical pattern of autolysis (Trump and Ginn, 1969).

In contrast, Trowell (1966) working with rat thymocytes and lymph node lymphocytes in organ culture, reported glucocorticoid-induced morphological changes typical of what he termed 'radiomimetic lesion'. The nuclear envelope disappears and the cytoplasm darkens in appearance due to an increase in the number of ribosomes. The cell membrane then disrupts and the nucleus shrinks and coarse and dark/...

dark cytoplasmic debris remain. In lymph node lymphocytes there is also a darkening in appearance of the nucleus at an early stage accompanied by lobulation and shrinking. These changes closely resemble apoptosis (Kerr, Wyllie and Currie, 1972 - see fig. 2, p. 4) and indeed apototic cells have also been observed more recently by Wyllie (in preparation) after in vitro treatment of rat thymocytes with a lethal concentration of glucocorticoid.

However, little work has involved the study of morphological changes in glucocorticoid-induced cell death in human lymphoid cells in vitro, although the morphological changes associated with sub-lethal injury have been described (Werthamer and Amaral, 1975). This section therefore deals with a study of morphological changes seen by both light microscope and electron microscope studies in $BLA_1$ cells over a 48hr incubation period with a lethal concentration ($1.4 \times 10^{-3}$M) of MPS. I also carried out a similar light microscope study on RAJI cells and the glucocorticoid-induced morphological changes were essentially the same as for $BLA_1$ cells.

## MATERIALS AND METHODS

### Morphological studies

Cultures of $BLA_1$ cells resuspended in fresh growth medium were set up at a concentration of $2 \times 10^5$ cells/ml. After 24hr, to allow cells to become established in logarithmic growth phase, the cultures were treated with 1% (v/v) of stock MPS solution to give a final concentration of $1.4 \times 10^{-3}$M MPS. Control cultures were treated/...

treated with 1% (v/v) of sterile distilled water. Samples were removed at 0hr, 30 min., 1hr, 2hr, 3hr, 4hr, 6hr, 8hr, 12hr, 24hr and 48hr after treatment and prepared for light microscopy and transmission electron microscopy. For scanning electron microscopy, samples were removed at 30 min., 1hr, 2hr, 3hr, 4hr, 6hr and 12hr after treatment.

Cell smear preparation for light microscopy (LM)

2-5 x $10^5$ cells were centrifuged at 400 x g for 5 min. at room temperature. The supernatant was discarded leaving a few drops in which the cells were resuspended. The cells were then smeared onto glass slides as shown in fig. 12. This method separates out the cells for good staining with Giemsa but keeps them at a fairly high density for easier counting. Smears were dried rapidly in a cold air stream, fixed in 95% methanol for 30 min., stained with 4% (v/v) Giemsa (Gurr's Improved R66, Searle Diagnostic) for 10 min. and then mounted in Harleco synthetic resin (Harleco).

Cell preparation for transmission electron microscopy (TEM)

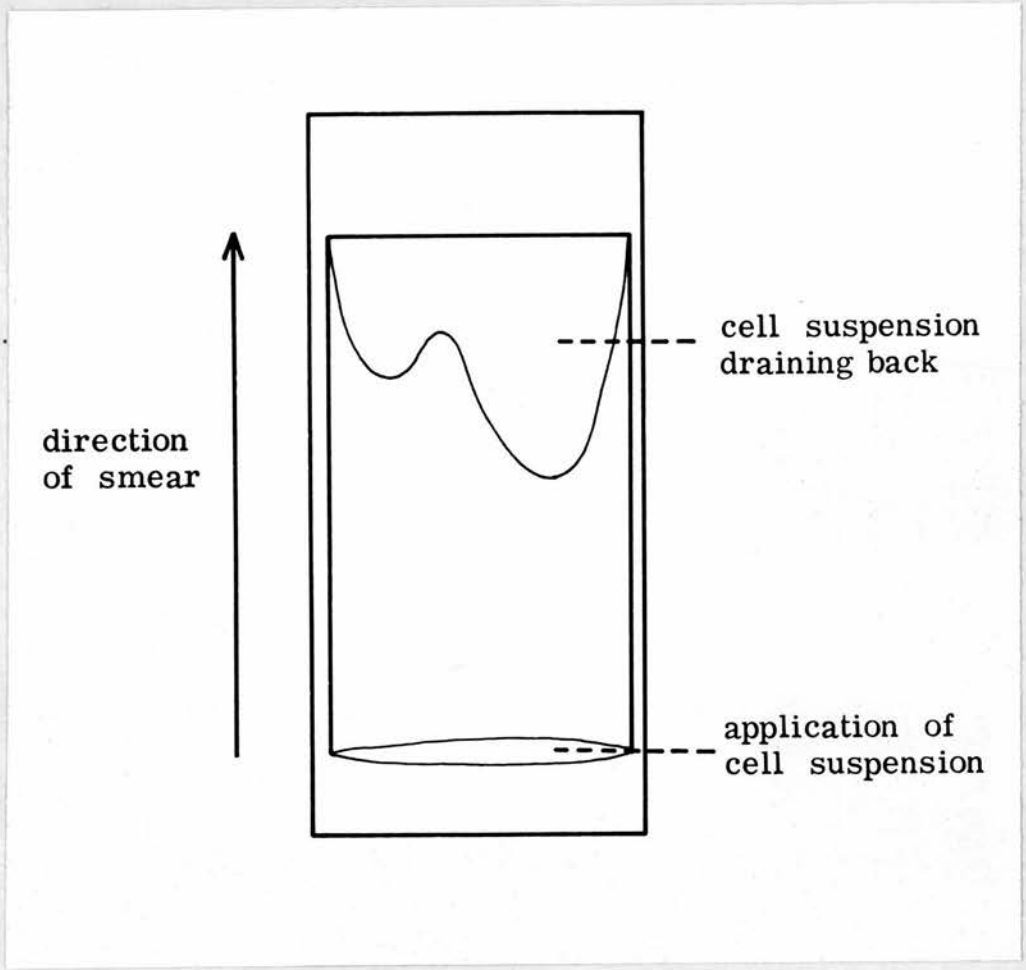
50-100 x $10^5$ cells were centrifuged at 400 x g for 5 min. at room temperature in a sterile plastic universal container (Sterilin), and the resulting pellet resuspended in 1ml of reconstituted sterile frozen-dried human plasma. All subsequent procedures, unless otherwise mentioned, were carried out at $4^{\circ}C$. Three % gluteraldehyde (TAAB) in 0.2M sodium cacodylate buffer (BDH) at pH7 was added, the suspension fixed for 1hr and then centrifuged at 2,000 x g for 5 min. to form a compact pellet in the cone of the universal container. After further fixation for 2hr (to allow cross-linking of the protein

to/...

Fig. 12

Preparation of cell smears

Cells are smeared onto a glass slide by applying a drop of cells at one end of the slide, drawing this out along most of the length of the slide with a second slide, then allowing the suspension to drain back on the first slide by standing it on end.



to form a fairly cohesive cell pellet), the pellet was washed in 0.2M sodium cacodylate buffer for 2hr and then post-fixed for 1hr in 1% osmium tetroxide ($OsO_4$) (Johnson Matthey) in 0.2M sodium cacodylate buffer. After 30 min. in $OsO_4$, the pellet was scraped gently from the cone of the universal container with a sharpened wooden spatula, to allow full penetration of the sides of the pellet with $OsO_4$ for a further 30 min.; this resulted in fragmentation of the pellet. The fragments were washed in 0.2M sodium cacodylate buffer for 30 min. and then dehydrated through a graded alcohol series allowing 20 min. for each change: 20%, 40%, 70%, 95% and three times in absolute ethanol. Fragments were transferred to glass phials and resuspended twice in epoxy-propane (BDH) at room temperature and then impregnated and set in epon resin (TAAB). Thin sections were cut on an LKB Ultrotome I with glass knives and mounted on formvar (TAAB) coated grids (Athene-type old 400). These were stained with saturated uranyl acetate (BDH) in 50% ethanol for 15 min., followed by 0.04M lead citrate (BDH) in 0.02M sodium hydroxide for 5 min. Sections were viewed on an AEI Corinth 275 electron microscope.

Cell preparation for scanning electron microscope (SEM)

(This work was undertaken by Andrew Waddell.) The cells were centrifuged gently (250 x g for 5 min.) and resuspended at $50 \times 10^5$ cells/ml in the medium in which they were grown. One drop of this suspension was placed at either end of a 6 x 35 mm coverslip and allowed to settle for 30 min. The cells were fixed by addition of one drop of 1% $OsO_4$ in 0.2M cacodylate buffer, and after 30 min. were gradually dehydrated by passing through increasing concentrations of/...

of acetone, and stored in 100% acetone. The samples were dried by critical point drying, which involved the substitution of acetone with liquid $CO_2$ and raising the temperature until it was above the critical point at which there is instantaneous evaporation of the $CO_2$. After coating with gold, the samples were viewed in a Cambridge Stereoscan 180 microscope.

## RESULTS

LM and TEM observation on the lethal glucocorticoid response

Control cultures. $BLA_1$ cells were round, oval or elongated with occasional cytoplasmic protrusions and long slender surface processes; cytoplasmic vacuoles were common (fig. 13). Ultrastructurally, the cytoplasm was rich in free polysomes and contained scanty profiles of rough endoplasmic reticulum, mitochondria with plate-like cristae, golgi apparatus and lipid droplets (fig. 14). Cells showed a high nuclear to cytoplasmic ratio and the nuclei, usually one per cell, were of variable shape and size with chromatin dispersed diffusely throughout the nucleoplasm and with one or more nucleoli (figs. 13 and 14). Scanty aggregates of heterochromatin were seen round the periphery of the nucleus (fig. 14).

Small numbers of cells showing necrotic changes were also observed (vide infra, see p. 59).

Treated cultures. No significant differences were observed from control cultures until 1hr after incubation with $1.4 \times 10^{-3}$M MPS

(see/...

Fig. 13

Control $BLA_1$ cells

The cell surface shows cytoplasmic protrusions (P) with numerous long slender surface processes (SP), and the cytoplasm contains many clear vacuoles (V). The cells have a high nuclear to cytoplasmic ratio, with the nuclei (N) showing diffusely dispersed chromatin. Giemsa (G). x 1,800.

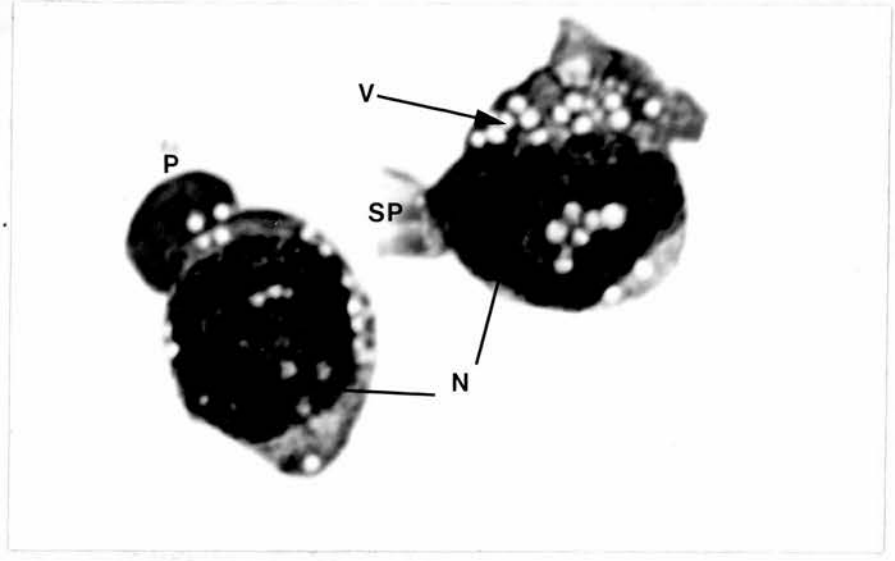
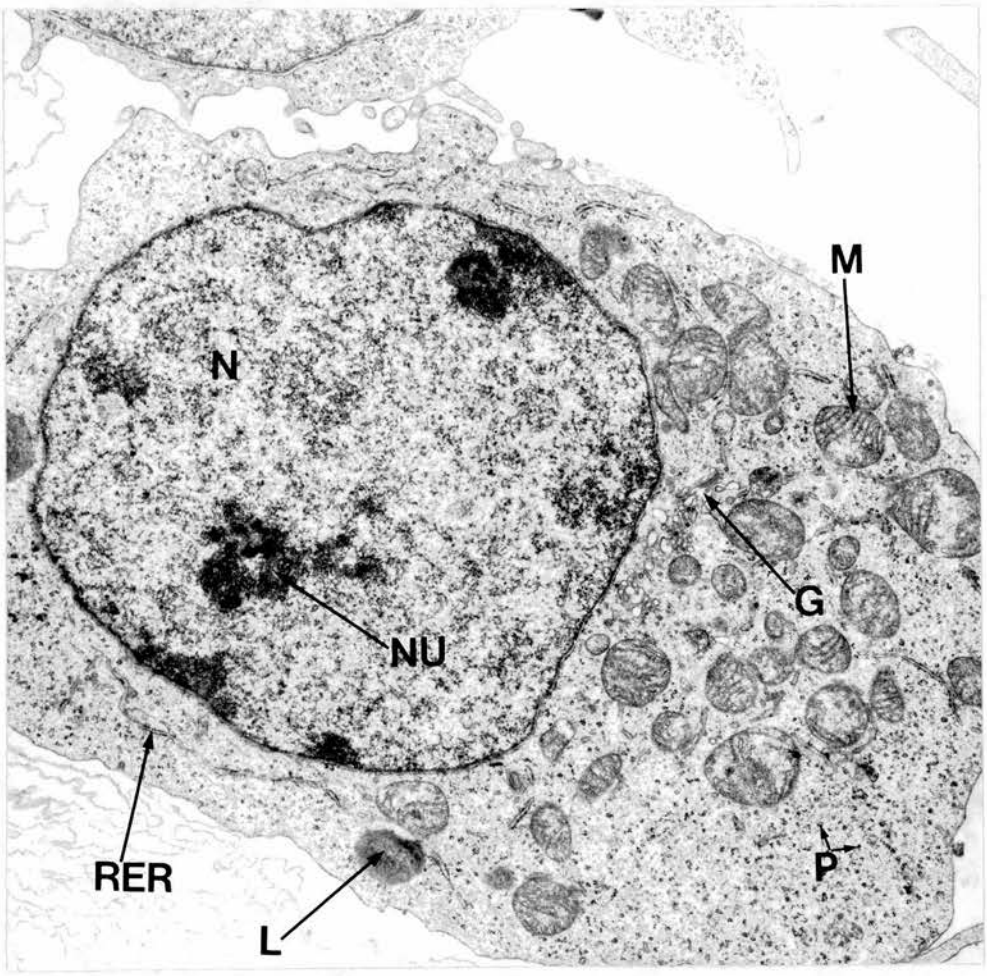


Fig. 14

Transmission electron micrograph (TEM) of control $BLA_1$ cell

The cytoplasm is rich in polysomes (P) with scanty profiles of rough endoplasmic reticulum (RER), mitochondria with plate-like cristae (M), golgi apparatus (G), and lipid droplets (L). The nucleus (N) shows diffusely dispersed chromatin with a prominent nucleolus (NU). Some scanty aggregates of heterochromatin are seen along the nuclear membrane. Uranyl acetate and lead citrate (UALC). x 10,400.



(see table 5, p. 48) when two distinctive morphological changes were seen.

The first type of change, whose incidence was markedly increased from 1 to 6hr after treatment with a peak at 2hr (table 5, p. 48), involved 'blebbing' of the cells. The cells showed loss of long slender surface processes with contortion and blebbing of the cytoplasm and indentation of the nucleus (figs. 15-17) which sometimes showed pyknosis and fragmentation (figs. 15 and 17). The 'blebbing' cells then began to fragment into discrete membrane-bounded cytoplasmic bodies of variable size (fig. 18), and were then seen as a cluster of membrane-bounded fragments (fig. 19), some of which still contained a few structurally recognisable organelles and nuclear fragments with or without nuclear membrane breakdown. In this example (fig. 19) the nuclear membrane still appears to be intact and there is no evidence of pyknosis.

By contrast, the second type of change, whose incidence was increased from 1 to 48hr after treatment with a peak at 4hr (table 5, p. 48) involved 'rounding up' of the cells. These cells showed loss of protrusions and long slender surface processes and this was accompanied by marked nuclear pyknosis, nuclear membrane breakdown and sometimes nuclear fragmentation (figs. 20-24). In smears, as well as showing nuclear changes, the cytoplasm became intensely stained and vacuoles appeared to congregate together within the cytoplasm (figs. 20 and 21). This was confirmed by TEM (figs. 22 and 23) which demonstrated aggregation of cytoplasmic organelles; several/...

TABLE 5 PERCENTAGE MORPHOLOGICAL TYPES IN SMEAR PREPARATIONS OF $BLA_1$ CELL CULTURES DURING TREATMENT WITH $1.4 \times 10^{-3}$M METHYLPREDNISOLONE OVER 48HR

| Time after treatment (hr) | Percentage* morphological type ± 1S.E. | | | |
|---|---|---|---|---|
| | Normal | 'Blebbing' | 'Rounding up' | Degenerate cells and degenerate fragments with nuclear remnants |
| 0 | 93.5 ± 1.2 | 0.1 ± 0.1 | 0.5 ± 0.1 | 5.9 ± 1.2 |
| 0.5 | 94.8 ± 1.3 | 0.1 ± 0.1 | 0.4 ± 0.1 | 4.7 ± 1.3 |
| 1 | 90.1 ± 2.2 | 2.6 ± 1.2 | 1.6 ± 0.5 | 5.7 ± 0.8 |
| 2 | 79.1 ± 4.8 | 4.6 ± 1.4 | 7.5 ± 2.2 | 8.8 ± 1.6 |
| 3 | 71.8 ± 8.1 | 3.2 ± 1.0 | 12.1 ± 3.3 | 12.9 ± 4.0 |
| 4 | 67.4 ± 7.1 | 2.9 ± 0.6 | 14.4 ± 3.4 | 15.3 ± 3.5 |
| 6 | 62.6 ± 9.6 | 1.2 ± 0.2 | 11.0 ± 2.1 | 25.2 ± 7.9 |
| 8 | 58.9 ± 8.4 | 0.6 ± 0.2 | 8.4 ± 2.0 | 32.1 ± 8.9 |
| 12 | 58.0 ± 11.3 | 0.6 ± 0.2 | 5.5 ± 1.3 | 35.9 ± 11.0 |
| 24 | 36.8 ± 11.6 | 0.2 ± 0.1 | 3.7 ± 1.3 | 59.3 ± 11.2 |
| 48 | 3.9 ± 1.4 | 0.1 ± 0.1 | 4.3 ± 2.8 | 91.7 ± 4.1 |
| control† | 92.9 ± 0.7 | 0.3 ± 0.1 | 0.8 ± 0.4 | 6.0 ± 0.5 |

*1000 cells scored per smear. Each value represents the mean ± 1S.E. of 5 separate experiments.

†control percentages represent the mean ± 1S.E. of counts at 0hr and 48hr after treatment with water.

Fig. 15

BLA$_1$ cells 2hr after treatment with 1.4 x $10^{-3}$M methylprednisolone

The cell (arrowed) shows marked cytoplasmic and nuclear blebbing. The nucleus shows pyknosis and fragmentation. G. x 1,600.

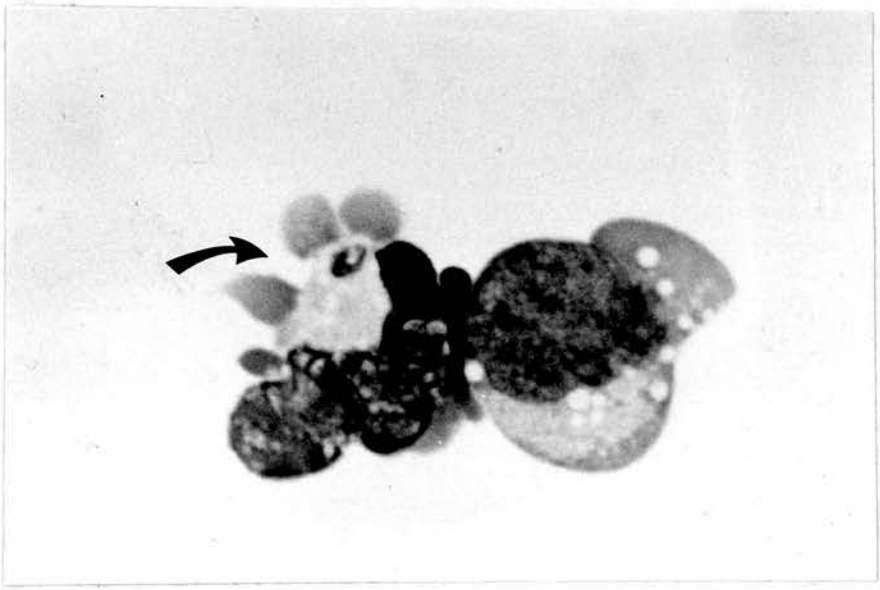


Fig. 16

TEM of $BLA_1$ cell 1hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell shows marked cytoplasmic blebbing and slight blebbing of the nucleus. UALC. x 10,400.

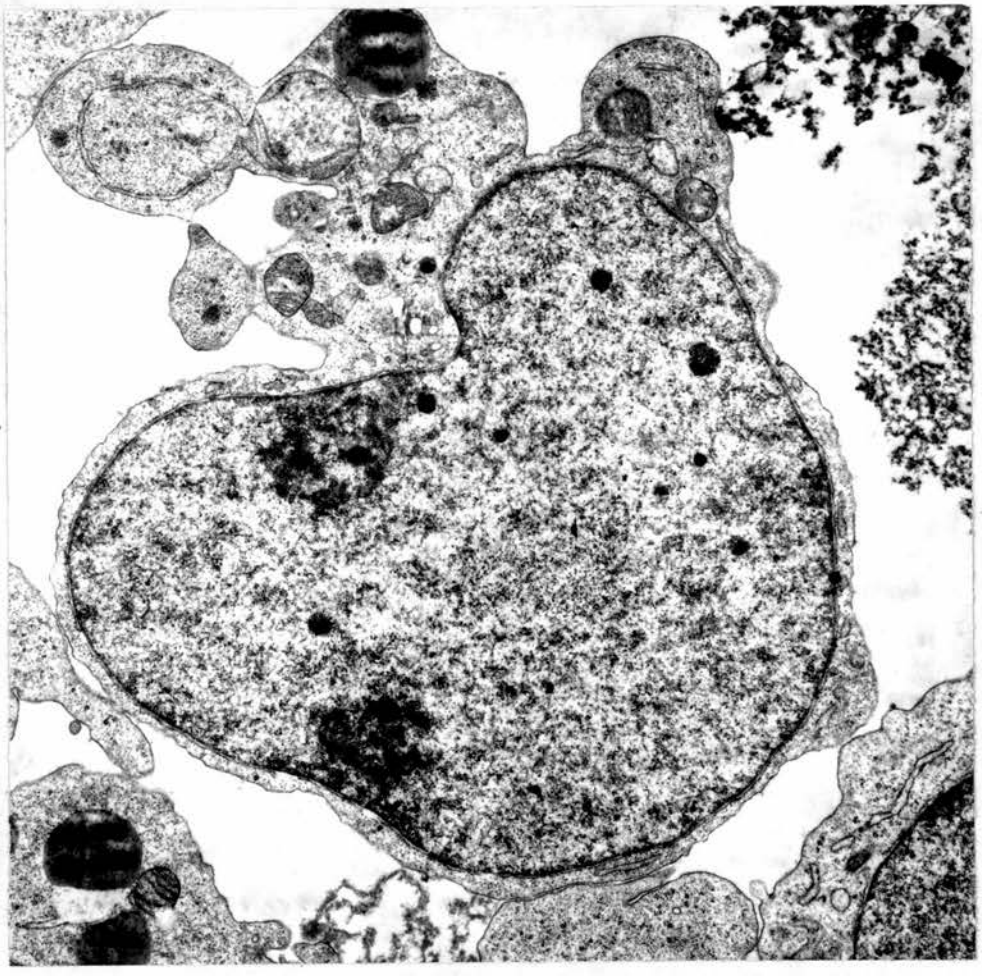


Fig. 17

TEM of $BLA_1$ cell 2hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell shows marked cytoplasmic blebbing, and nuclear indentation and fragmentation. UALC. x 16,000.

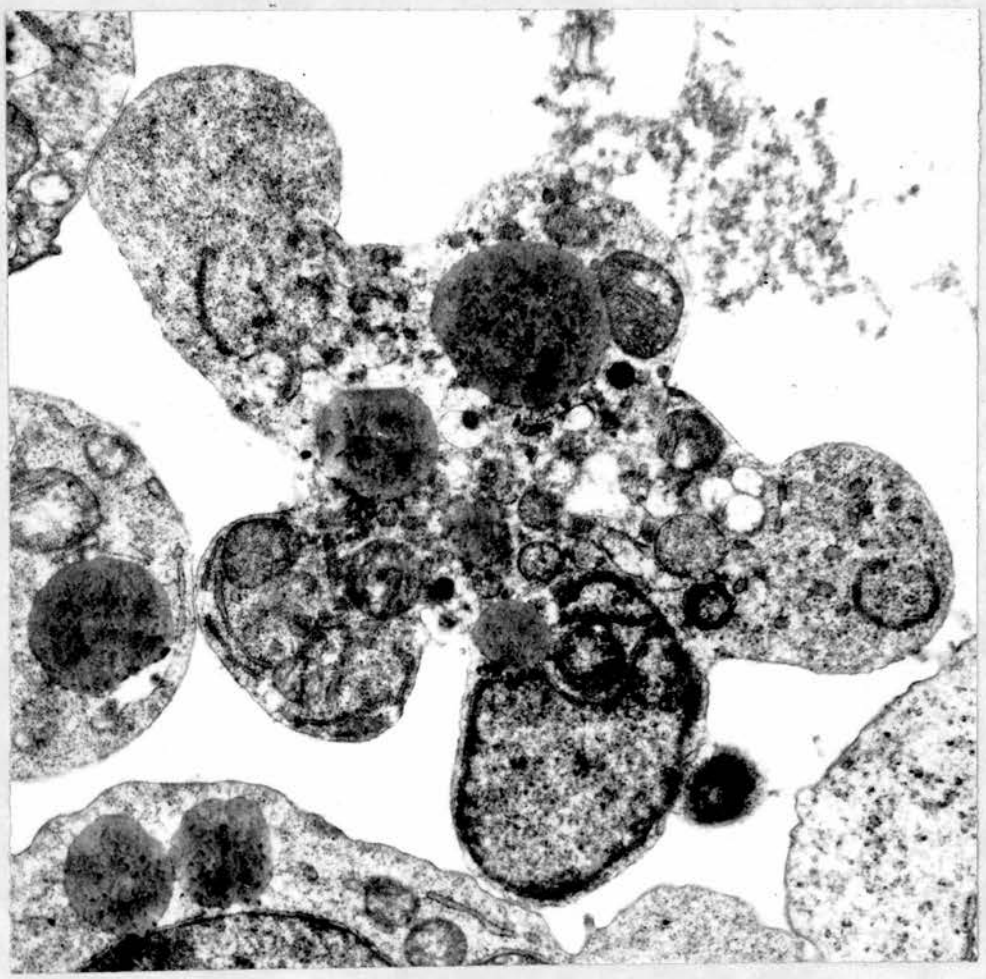


Fig. 18

TEM of $BLA_1$ cell 1hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell shows marked cytoplasmic blebbing with fragmentation to form membrane-bounded fragments; cytoplasmic organelles are still intact. UALC. x 6,250.

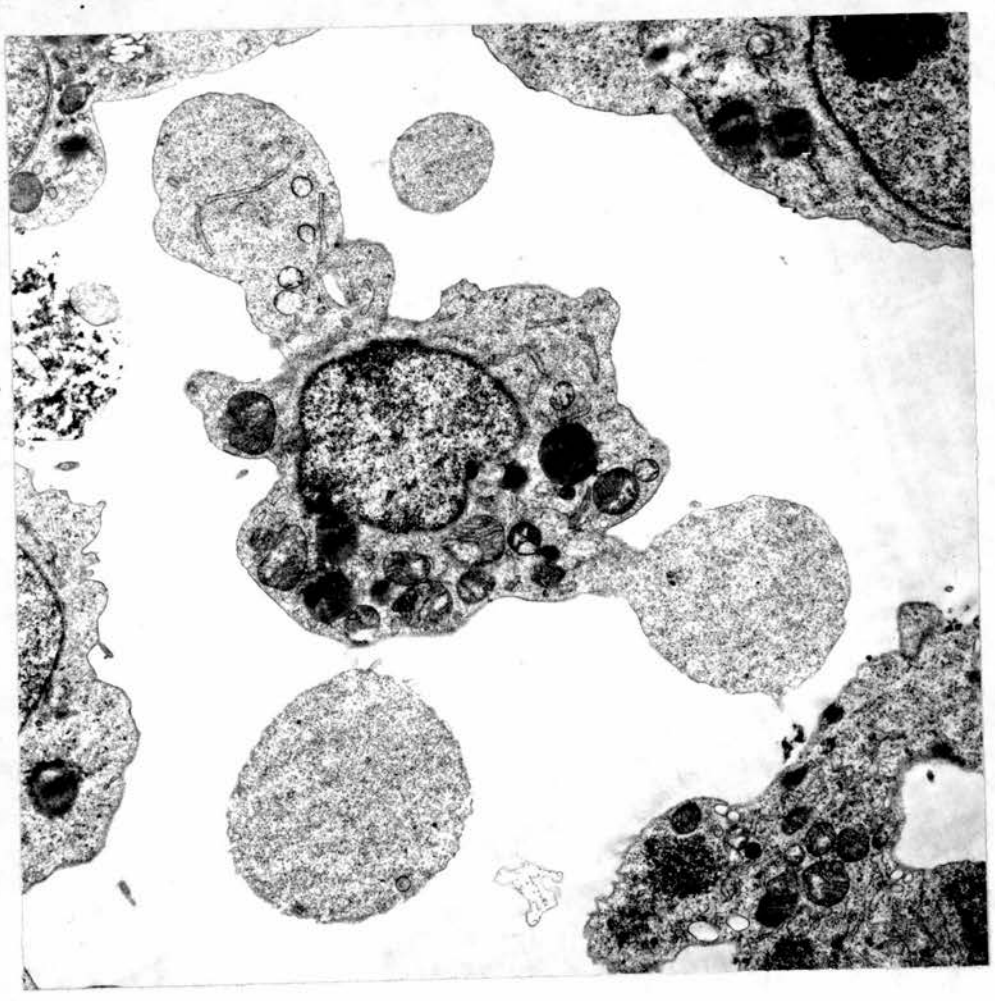


Fig. 19

TEM of $BLA_1$ cell 1hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell is represented by a cluster of membrane-bounded fragments, most of which contain structurally recognisable organelles and one of which contains nuclear material (arrowed). UALC. x 10,400.

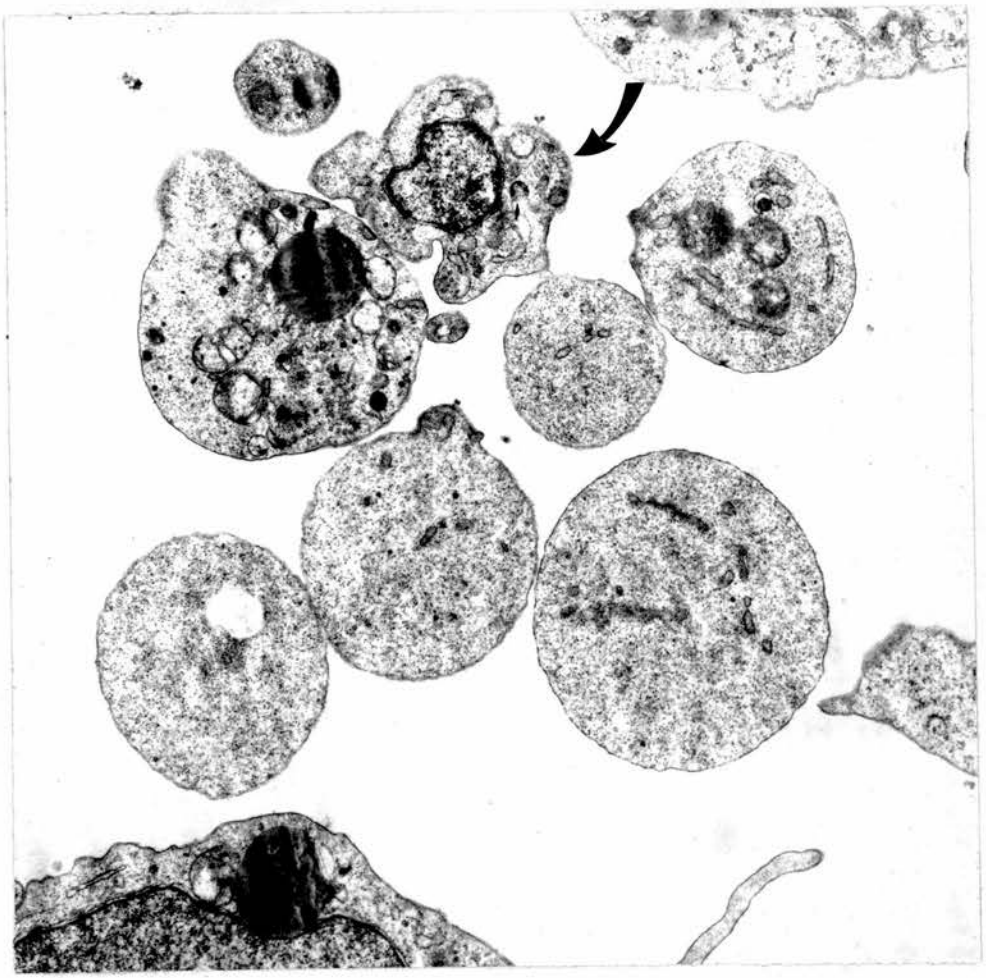


Fig. 20

$BLA_1$ cells 1hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell (arrowed) shows rounding up with loss of surface protrusions and slender surface processes. The cytoplasm contains aggregated vacuoles, and the nucleus shows the initial chromatin condensation pattern of pyknosis. G. x 1,800.

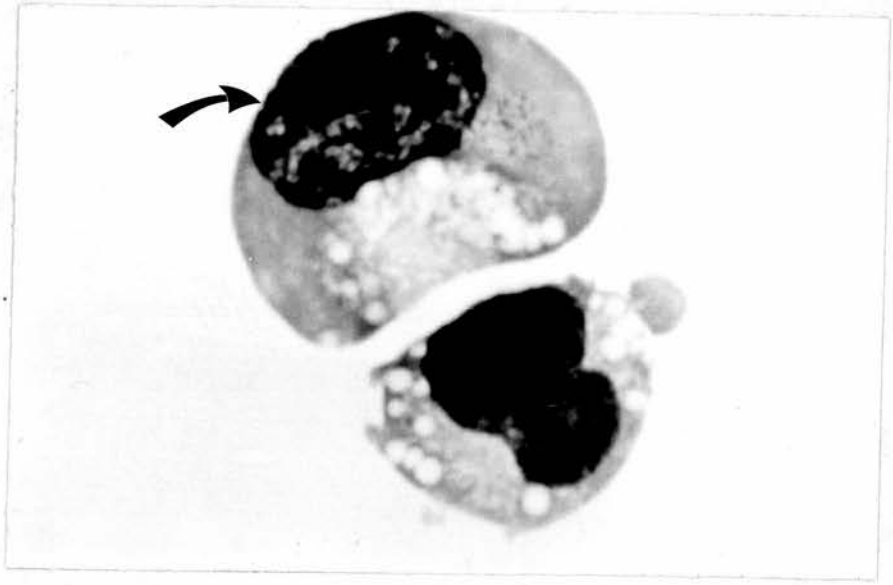


Fig. 21

$BLA_1$ cells 4hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cells show rounding up with loss of surface protrusions and slender surface processes. The cytoplasm contains aggregated vacuoles. The nuclei show pyknosis and fragmentation. G. x 1,540.

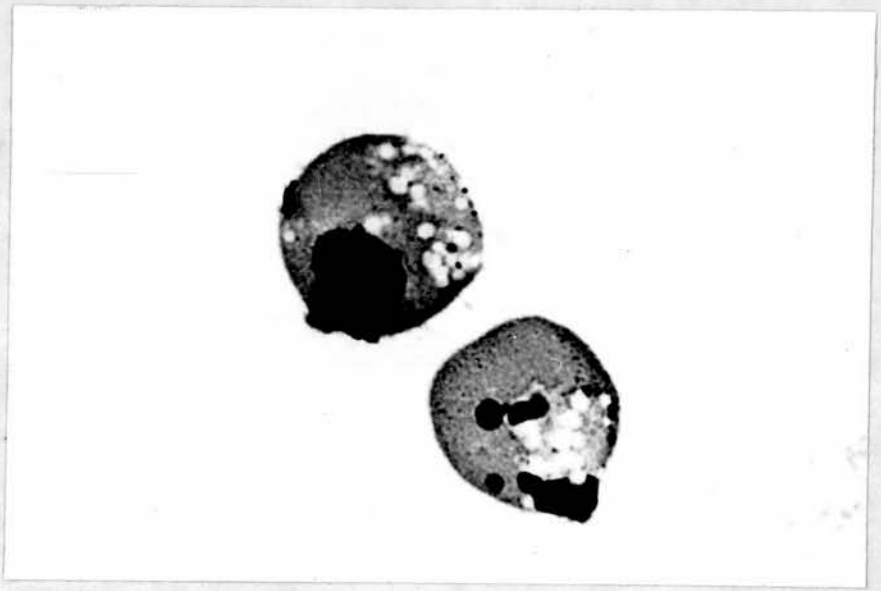


Fig. 22

TEM of $BLA_1$ cell 6hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell shows rounding up with loss of surface protrusions and slender surface processes, and cytoplasmic organelles are aggregated together. The nucleus shows the initial chromatin condensation pattern of pyknosis. UALC. x 10,400.

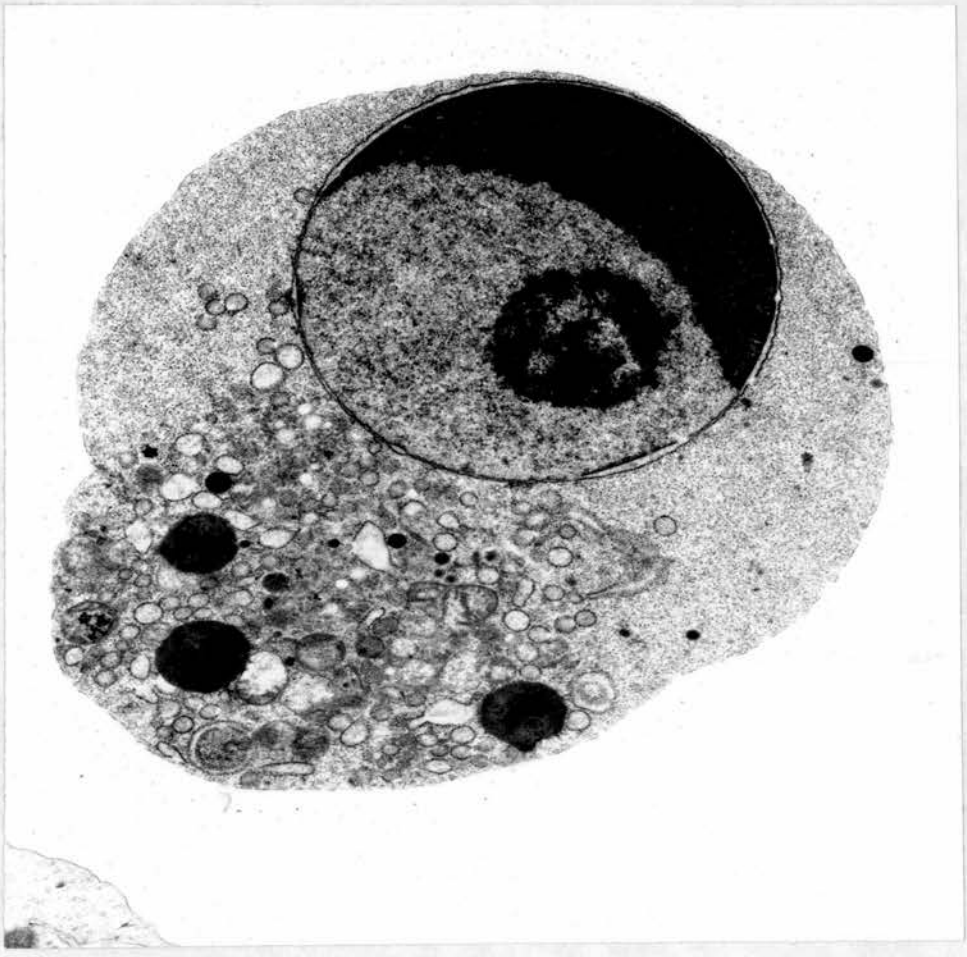


Fig. 23

TEM of $BLA_1$ cell 6hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell shows rounding up with loss of surface protrusions and slender surface processes, and cytoplasmic organelles are aggregated together. The nucleus shows pyknosis with nuclear membrane breakdown. UALC. x 10,400.

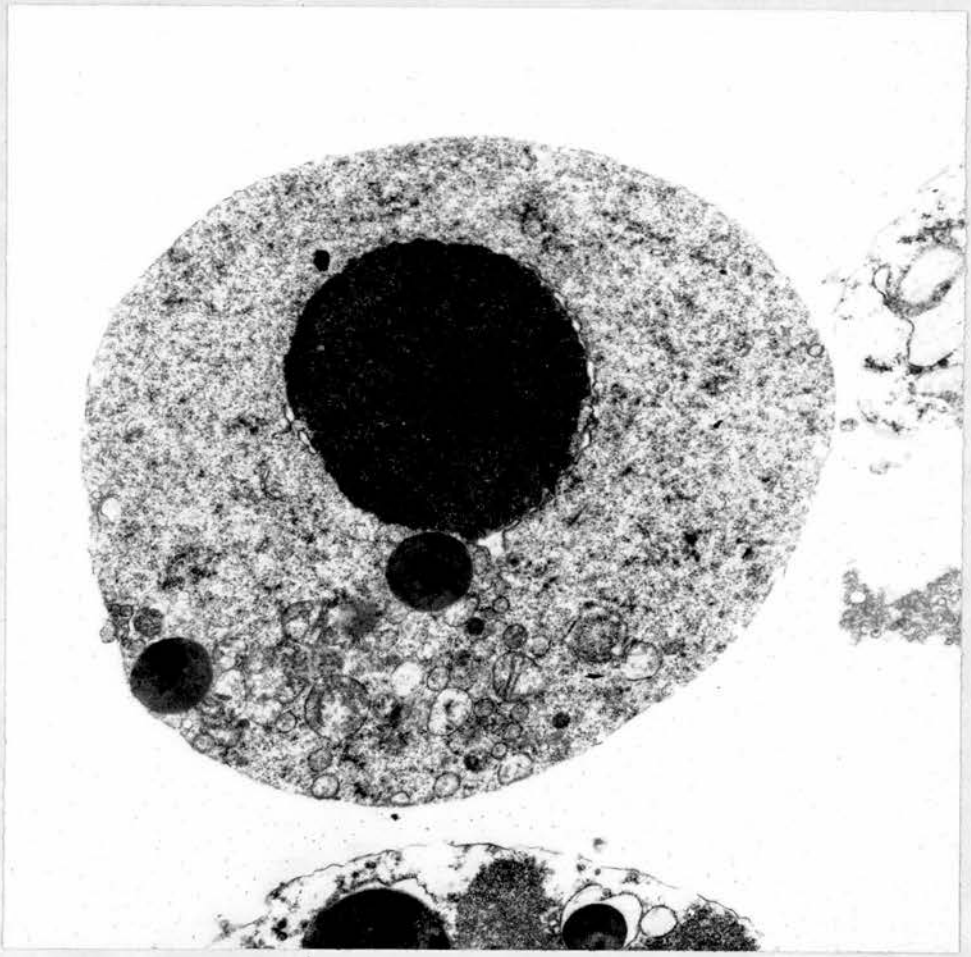
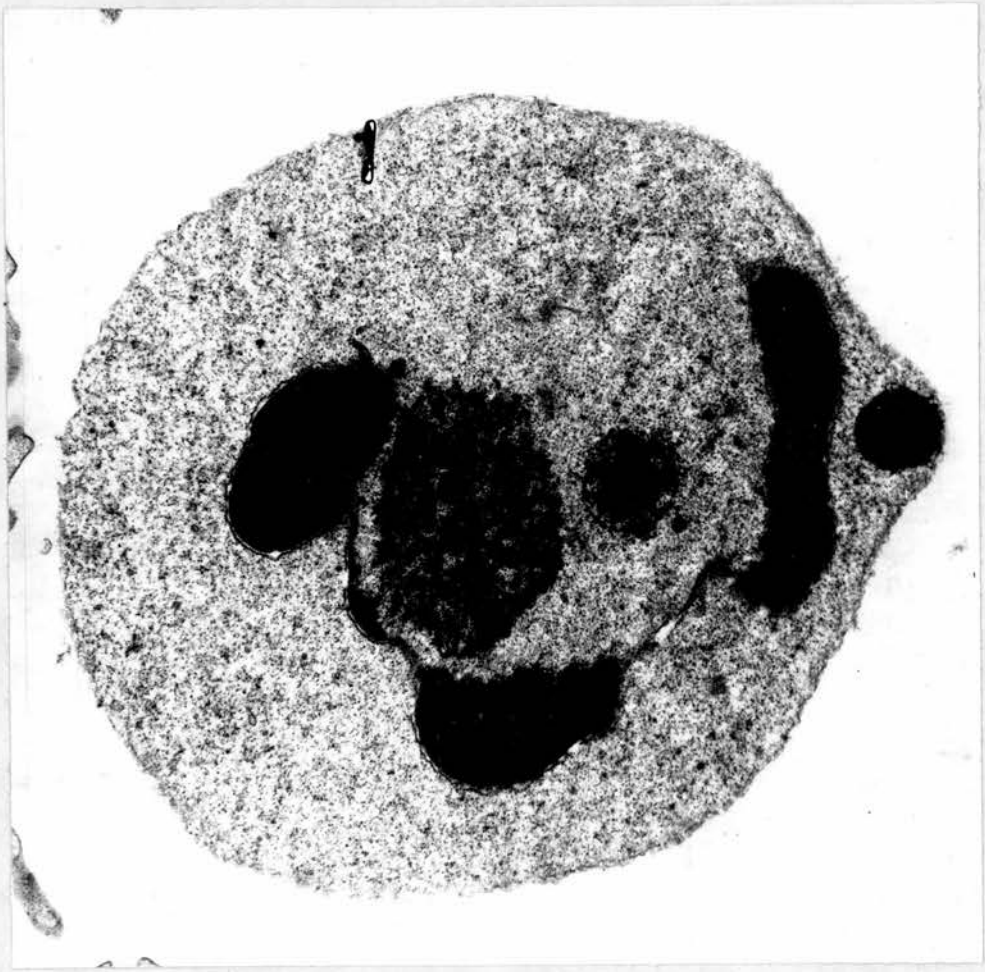


Fig. 24

TEM of $BLA_1$ cell 4hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell shows rounding up with loss of surface protrusions and slender surface processes. The nucleus shows pyknosis, fragmentation and nuclear membrane breakdown. (This section is through the part of the cell which contains no aggregated cytoplasmic organelles.) UALC. x 11,900.



several mitochondria with cristae were occasionally visible.

Whilst these were the earliest morphological changes, from 2-3hr onwards after treatment an increasing percentage of cells and fragments began to show more marked degenerative changes (table 5, p. 48), and at 48hr over 90% showed features of autolysis (Trump and Ginn, 1969). In smears, these cells were recognised by their diminished intensity of nuclear and cytoplasmic staining. Ultrastructural features typical of this late-stage process of cell death included focal dissolution of the plasma membrane and breakdown of cytoplasmic organelles, distended mitochondria with flocculent matrix densities and loss of cristae and further dissolution of the fragmented nucleus, the nucleoplasm becoming increasingly electron lucent (fig. 25).

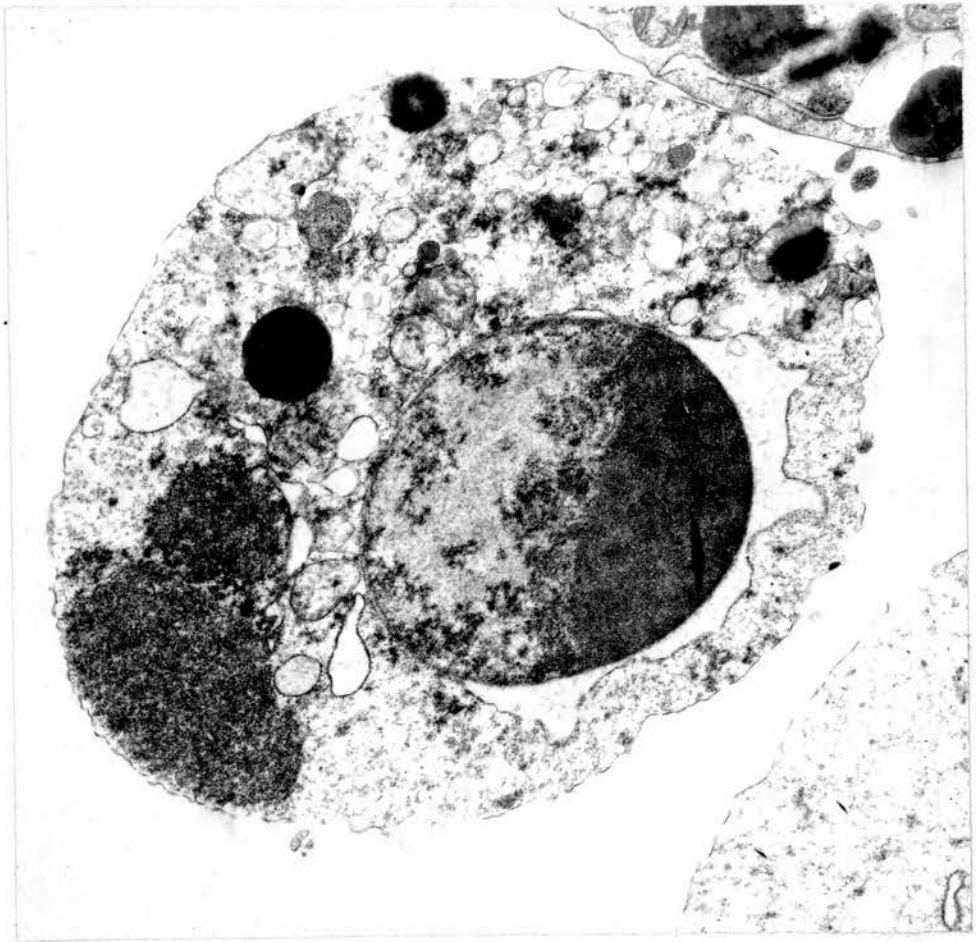
About 4% of the cells appeared to have survived MPS treatment and retained normal morphology 48hr after treatment (table 5, p. 48).

Small numbers of dying cells were also seen in control culture smears (table 5, p. 48). Some of these cells contained aggregated vacuoles and pyknotic nuclear fragments; some appeared as 'blebbing' cells or cellular fragments with or without nuclear remnants; and some showed changes typical of autolysis. These observations suggest that death by both 'rounding up' and 'blebbing', followed by further degenerative changes, were also occurring spontaneously in untreated cultures. Ultrastructurally, changes typical of autolysis were also observed at a low frequency, and an occasional cell also showed dilatation of the endoplasmic reticulum and golgi apparatus, with indentation/...

Fig. 25

TEM of $BLA_1$ cell 4hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell shows marked degenerative changes with focal dissolution of the plasma membrane and loss of structure of cytoplasmic organelles. The pyknotic and fragmented nucleus is becoming electron lucent. UALC. x 16,000.



indentation of the nucleus and distension of the nuclear envelope (fig. 26).

SEM observations on the lethal glucocorticoid response

Control $BLA_1$ cells were basically round with a 'ruffled' surface, and occasionally processes were seen adhering to the substrate (fig. 27). After addition of $1.4 \times 10^{-3}$M MPS, there was an increase in the number of cells showing 'blebbing' on an otherwise smooth cell surface (fig. 28) and this reached a peak 2-3hr after treatment. Cells were examined up to 12hr after treatment, over which period there was a gradual increase in the number of small, subcellular particles in the samples, and of cells with an irregular surface morphology (fig. 29) which presumably were 'rounding up' cells. A gradual accumulation of enlarged cells and cell fragments were also seen and these are undoubtedly autolytic.

## COMMENT

The $BLA_1$ cell line studied had morphological features typical of human lymphoid B-cell lines (Nilsson and Pontén, 1975; Parker et al, 1978).

In treated cultures, many of the morphological manifestations of the lethal response to $1.4 \times 10^{-3}$M MPS were consistent with apoptosis (Kerr, Wyllie and Currie, 1972). The features are also similar to 'popcorn' and 'ballooning' processes of cell death described in HeLa cells after treatment with lymphotoxin (Russell, Rosenau/...

Fig. 26

TEM of dying $BLA_1$ cell in control culture

The cell shows dilatation of the endoplasmic reticulum and golgi apparatus. The nucleus shows indentation and fragmentation and distension of the nuclear envelope.
x 10,400.

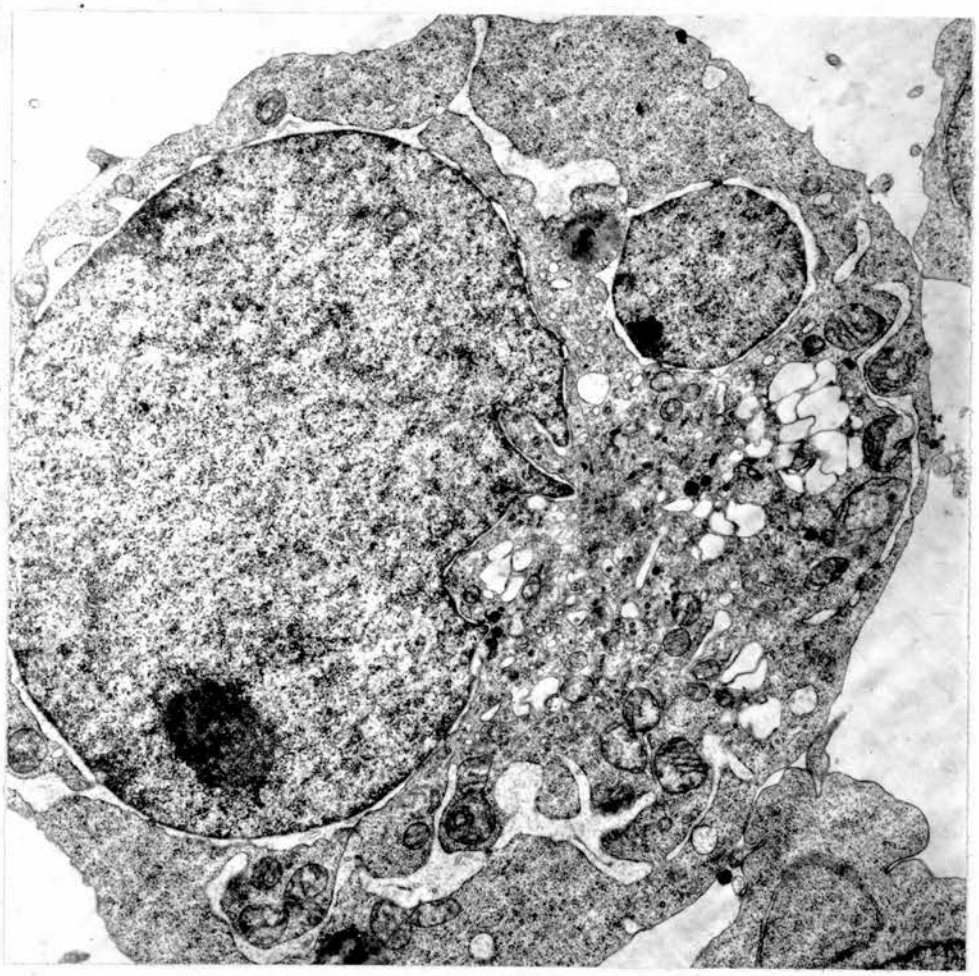


Fig. 27

Scanning electron micrograph (SEM) of control $BLA_1$ cell

The cell shows a 'ruffled' surface with processes adhering to the substrate. x 12,600.

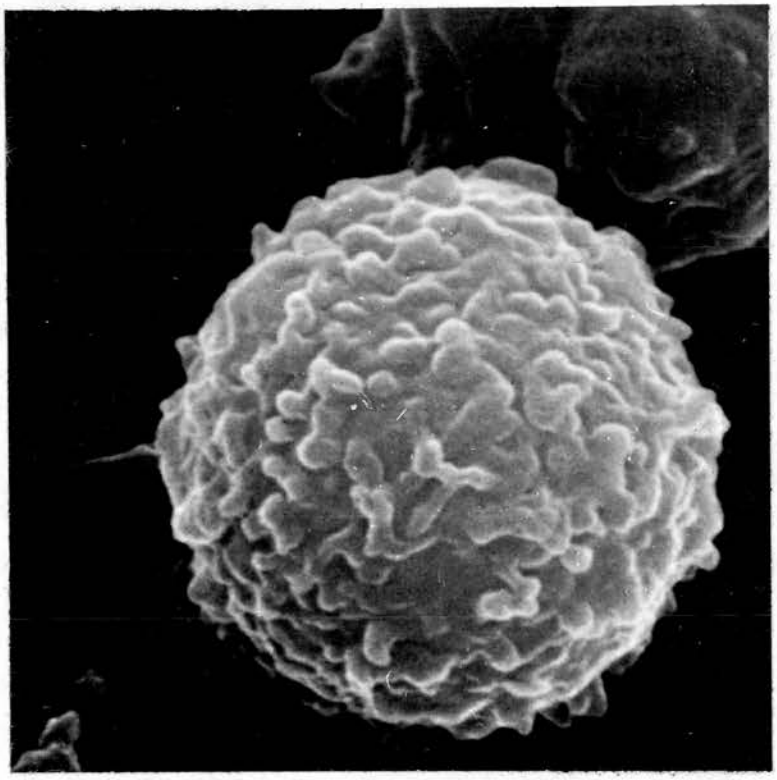


Fig. 28

SEM of $BLA_1$ cell 4hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell shows 'blebbing' on an otherwise smooth cell surface. x 7,650.

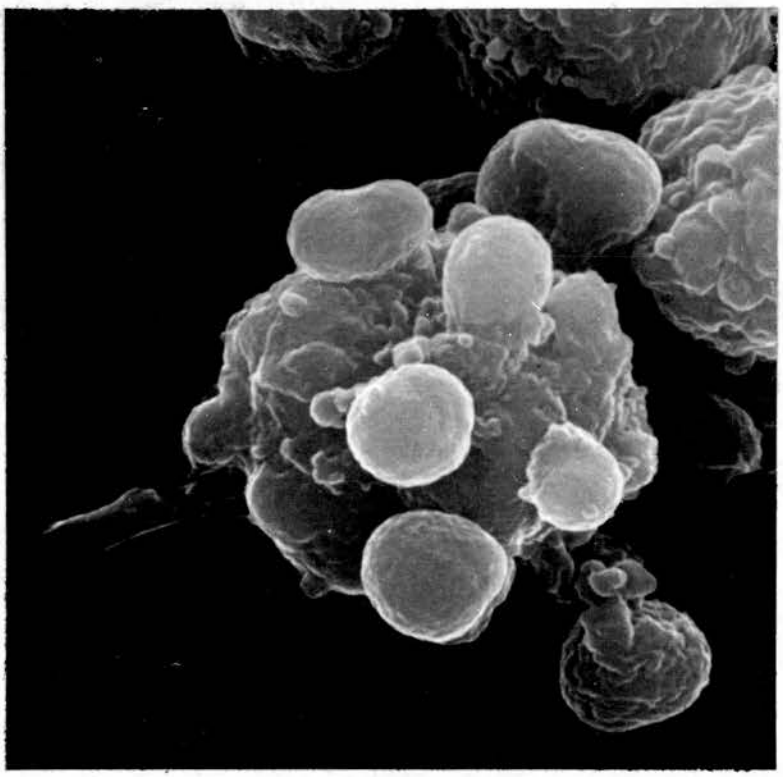
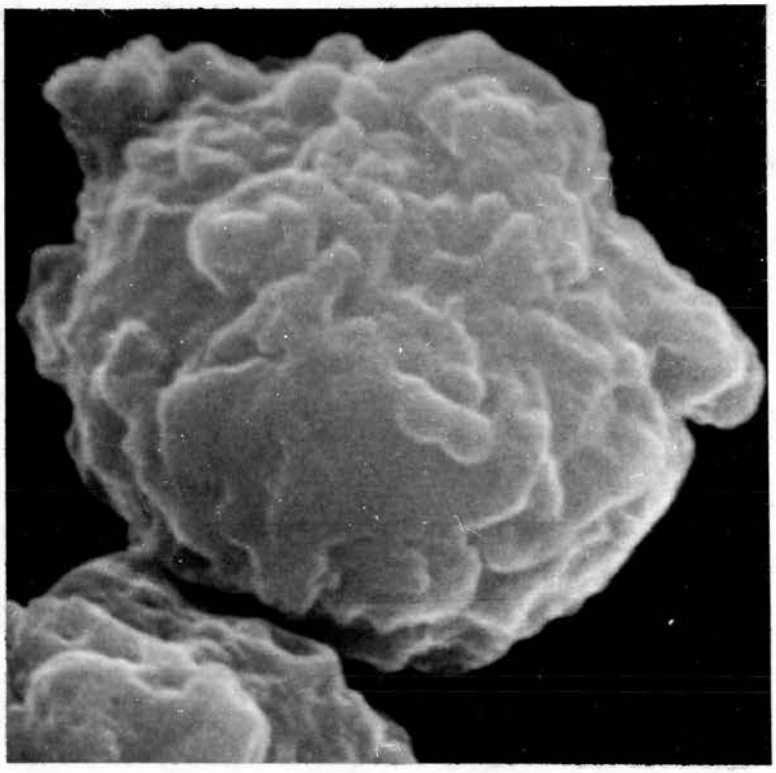


Fig. 29

SEM of $BLA_1$ cell 4hr after treatment with $1.4 \times 10^{-3}$M methylprednisolone

The cell shows an irregular surface. x 13,260.



Rosenau and Lee, 1972).

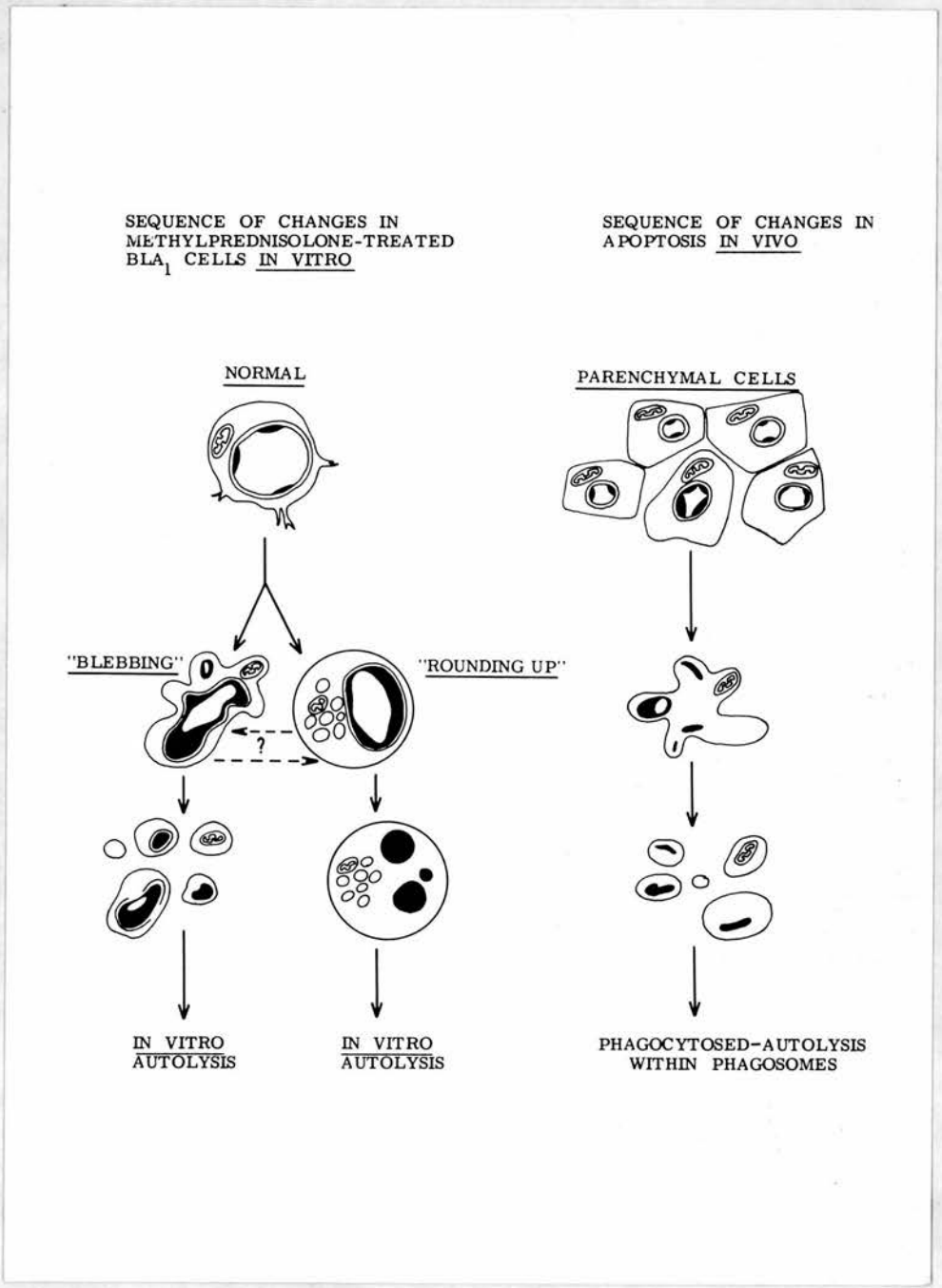
The sequence of morphological changes occurring both in our *in vitro* system and in apoptosis *in vivo* are summarised diagrammatically in fig. 30. In $BLA_1$ cells, from 1hr onwards after treatment with MPS, cells died by either 'blebbing' or 'rounding up'. 'Blebbing' cells showed contortion of the cytoplasm and nucleus resulting in the formation of discrete membrane-bounded fragments of variable size. These contained some apparently normal organelles, and nuclear fragments which showed pyknosis and nuclear membrane breakdown - in some cases nuclear pyknosis preceded cellular and nuclear fragmentation. All these changes occur in apoptosis *in vivo* (Kerr, Wyllie and Currie, 1972). The 'rounding up' cells also showed morphological changes typical of apoptosis: loss of cytoplasmic protrusions and long slender surface processes, preservation of structure of cytoplasmic organelles, nuclear pyknosis and fragmentation and nuclear membrane breakdown.

Both the 'blebbing' and 'rounding up' forms of cell death appear to have certain features in common, but from these studies it is not possible to say whether they are two distinct early changes or whether one form precedes the other (fig. 30 and see table 5, p. 48). We cannot claim that 'rounding up' precedes 'blebbing' which is the established sequence of events in apoptosis *in vivo*. However, it seems likely that the response of cells in suspension culture *in vitro* will differ in some respects from that of cells 'organised' *in vivo*.

From/...

Fig. 30

Diagrammatic summary of the sequence of morphological changes occurring both in methylprednisolone ($1.4 \times 10^{-3}$M) -induced cell death in $BLA_1$ cells in vitro and in apoptosis in vivo



From 3hr onwards after treatment, increasing numbers of dying cells and fragments showed further degradative changes typical of coagulative necrosis *in vivo* (Trump and Ericsson, 1965; Trump and Arstila, 1971) and of autolysis *in vitro* (Trump and Ginn, 1969) or post-mortem autolysis. Such degenerative changes are found in apoptosis *in vivo* after apoptotic bodies are phagocytosed by surrounding viable cells. However, it seems reasonable to suggest that in an *in vitro* system, in the absence of phagocytes, 'autolysis' would be expected to occur in apoptotic bodies. We suggest that the morphological changes demonstrated in our system are consistent with this sequence (fig. 30).

Certain *in vivo* studies also support such a possibility. In mouse ascites tumour cells treated with various non-steroidal cancer chemotherapeutic agents (Searle *et al*, 1975), many tumour cells which initially undergo apoptosis ultimately exhibit the features of coagulative necrosis while lying free in ascitic fluid, where apparently there are few active phagocytes. The same sequence of changes has also been noted in apoptotic cells shed into the glandular lumina of the involuting rat prostate in castration - induced atrophy (Kerr and Searle, 1973).

The morphology of spontaneous cell death in control cultures *in vitro* has received scant attention and it is of interest that we found precisely the same sequence of changes - albeit affecting only a small proportion of cells (table 5, p. 48 ) - as described after treatment with MPS.

Since/...

Since human lymphoid cells treated *in vitro* with glucocorticoids show a sequence of morphological changes closely similar to apoptosis *in vivo* then we believe that this provides a suitable system for studying the early biochemical events and intracellular control mechanisms involved in apoptosis. A correlation between the observed morphological changes and biochemical investigations is discussed later (see General Discussion, p. 157).

SECTION 3

## RELIABILITY OF NIGROSINE UPTAKE AS A MARKER OF GLUCOCORTICOID-INDUCED CELL DEATH IN HUMAN LYMPHOID CELLS

Exclusion of high molecular weight vital dyes such as nigrosine and trypan blue has been used classically as a light microscope marker in determining metabolic cell death (Schrek, 1936; Phillips and Terryberry, 1957; Kaltenbach, Kaltenbach and Lyons, 1958; Claesson, 1969). The method is based on a measure of the integrity of the plasma membrane, thus indicating cell membrane damage.

However there is controversy over the practical use of vital dyes in measuring cell death in vitro. Apart from the fact that dye uptake has been found to be extremely variable under different staining conditions (Black and Berenbaum, 1964), there is also well-documented evidence indicating non-correlation of the method when compared with other well-known markers of cell death. In experiments involving X-irradiation of rat thymocytes, Myers and De Wolfe-Slade (1964) showed that under certain conditions, pyknotic cells and loss of intracellular $K^+$ always preceded cellular uptake of dye. Medzihradsky and Marks (1975) on studies with rat lymphoid cells and Ehrlich ascites tumour cells, showed that in response to adverse experimental conditions such as exposure to heat, hypertonic conditions and metabolic inhibition, trypan blue uptake was the least responsive/...

responsive index of cell viability when compared with release of cellular lactic dehydrogenase and decrease in the intracellular ratio of $K^+/Na^+$. In proliferating populations of human lymphoid cells after treatment with cytotoxic drugs, dye uptake was found to be an insensitive marker for assessment of cell survival when compared with $^3$H-thymidine uptake and colony formation inhibition, the last marker being the most sensitive (Roper and Drewinko, 1976) - however this could be expected as both a decrease in $^3$H-thymidine uptake and colony formation inhibition could indicate either reduced proliferative capacity or metabolic cell death, whereas dye uptake only indicates metabolic cell death. In the same study, $^{51}$Cr release which again indicates metabolic cell death - radioactive chromate bound to protein is released as a function of cell damage - was also found to be as insensitive a marker as dye uptake.

To determine the sensitivity of nigrosine as a marker for glucocorticoid-induced cell death in our system, we have compared the kinetics of nigrosine uptake with the kinetics of morphological changes observed by light microscopy in $BLA_1$ and RAJI cells after treatment with a lethal concentration of MPS over a 48hr incubation period.

## MATERIALS AND METHODS

### Kinetic studies

Cultures of $BLA_1$ and RAJI cells resuspended in fresh growth medium were set up at a concentration of $2 \times 10^5$ cells/ml. After 24hr, to allow cells to become established in logarithmic growth phase, cultures/...

cultures were treated with 1% (v/v) of stock MPS to give a final concentration of 1.4 x $10^{-3}$M and 1 x $10^{-3}$M MPS for $BLA_1$ and RAJI cultures respectively. (Although different steroid concentrations were used to treat $BLA_1$ and RAJI cells these were both within the observed cytolethal dose range - see figs. 6 and 7, pp. 27 and 28; results are therefore comparable.) Control cultures were treated with 1% (v/v) of sterile distilled water. Samples were removed sequentially over a 48hr treatment period (see figs. 31 and 32) and (a) prepared for LM studies i.e., cell smear preparation (see Materials and Methods, p. 41), (b) assessed for viability, by the ability of live cells to exclude nigrosine (see Materials and Methods, p. 20).

## RESULTS

### Kinetics of the lethal glucocorticoid response

The kinetics of the glucocorticoid-induced morphological changes and the decrease in cell viability are shown in figs. 31 and 32 for $BLA_1$ and RAJI cells respectively.

(a) $BLA_1$ cells. The sequence of morphological changes was described in the previous section and fig. 31 shows plots of values for the morphological types presented in table 5 (p. 48). The cells described as 'blebbing' and 'rounding up' in table 5 are grouped in fig. 31 as 'apoptotic',and 'degenerate' cells are classed as 'autolytic'.

The percentage of apoptotic cells increased within the first hour after treatment reaching a peak of about 16% at 4hr after which it/...

Fig. 31

Kinetics of the morphological changes associated with the cytolethal response of $BLA_1$ cells to $1.4 \times 10^{-3}$M methylprednisolone (MPS)

Cultures were incubated with $1.4 \times 10^{-3}$M MPS (treated) or water (control) for 48hr and assessed over this period for: morphological change, as observed from smear preparations (1000 cells scored per smear); and viability, as measured by the ability of live cells to exclude nigrosine (200 cells scored per observation). ●——●, apoptotic cells; △——△, autolytic cells; O- - - -O, nigrosine stained cells.

Each point represents the mean of treated minus control values for 5 separate experiments. Control percentages were the means of counts at 0hr and 48hr.

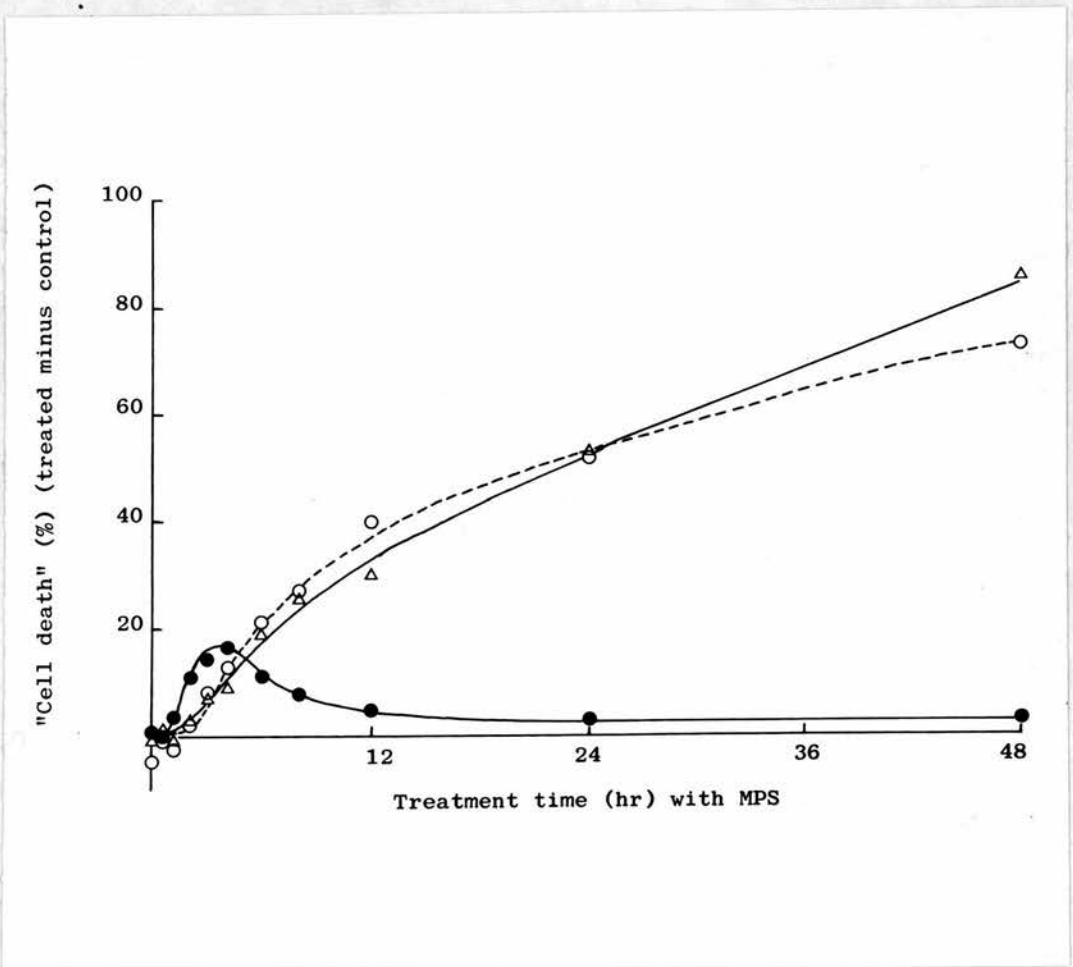
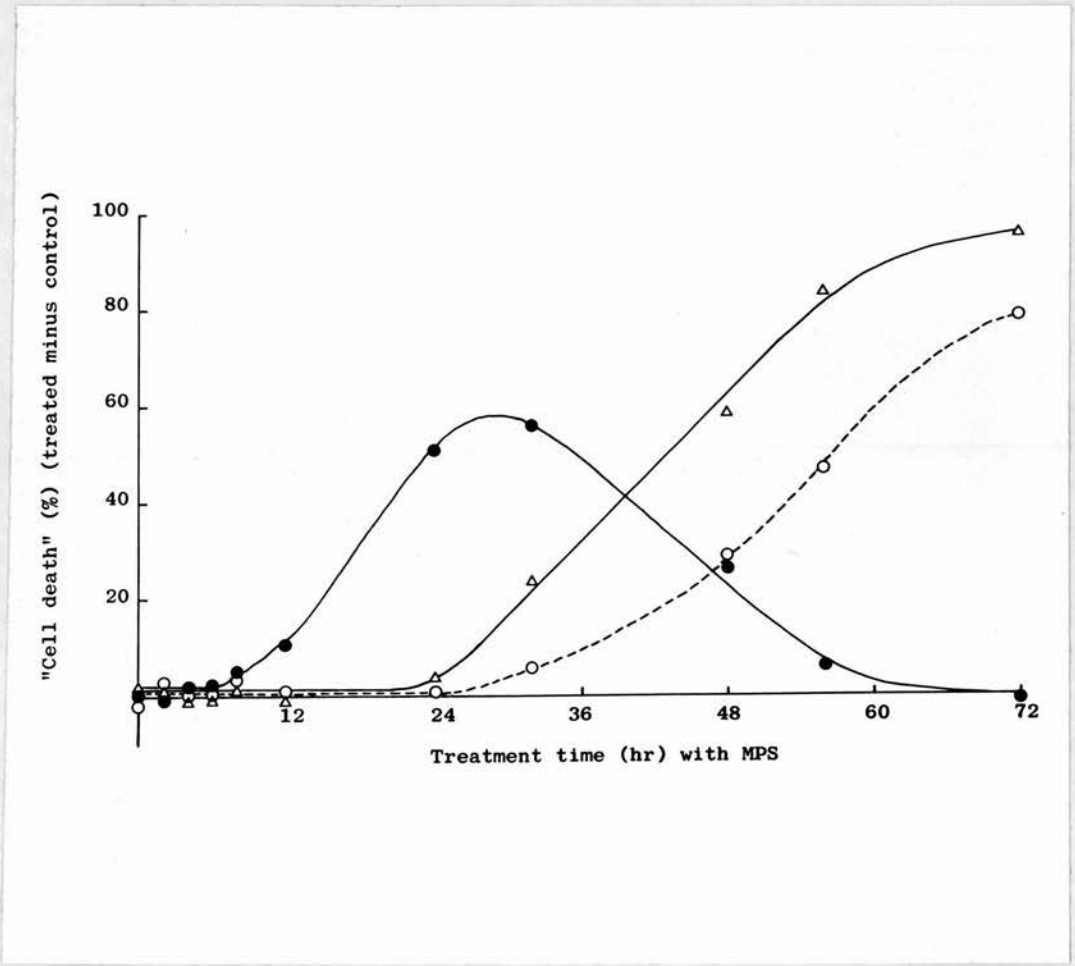


Fig. 32

Kinetics of the morphological changes associated with the cytolethal response of RAJI cells to 1 x $10^{-3}$M methyl-prednisolone (MPS)

Cultures were incubated with 1 x $10^{-3}$M MPS (treated) or water (control) for 48hr and assessed over this period for: morphological change, as observed from smear preparations (1000 cells scored per smear); and viability, as measured by the ability of live cells to exclude nigrosine (200 cells scored per observation). ●——●, apoptotic cells; △——△, autolytic cells; O- - - -O, nigrosine stained cells.

Each point represents the mean of duplicate tests from one experiment. Control percentages were the means of counts at 0hr and 72hr.



it declined to a plateau level of about 3% (fig. 31) - this reflects the transient existence of apoptotic cells which become autolytic. The percentage of autolytic cells increased exponentially after an initial lag phase of about 2hr reaching a level of about 85% by 48hr after treatment.

The percentage of nigrosine-stained cells followed approximately the same curve as for autolytic cells.

(b) RAJI cells. There was a delay of 6-8hr before onset of apoptosis (fig. 32). The percentage of apoptotic cells then increased to reach a peak of 58% at about 28hr after treatment and then fell again to its initial value. The percentage of autolytic cells increased exponentially after a lag phase of about 24hr reaching a peak of $> 95\%$ by 72hr after treatment.

Again, the percentage of nigrosine-stained cells followed a similar curve as for autolytic cells, though more delayed than in $BLA_1$ cells.

## COMMENT

The kinetic studies of the cytolethal response are essentially similar for $BLA_1$ and RAJI cells. However two differences are observed: in contrast to $BLA_1$ cells, RAJI cells showed a prolonged delay before onset of morphological changes - this is possibly due to the difference in MPS concentrations used ($1 \times 10^{-3}$M and $1.4 \times 10^{-3}$M MPS for RAJI and $BLA_1$ cells respectively) as the kinetics of/...

of the cytolethal response is dose-dependent (see fig. 10, p. 34); secondly, the percentage of apoptosis in RAJI cells reached a far higher peak than for $BLA_1$ cells which could reflect the existence of different 'metabolic states' between the cell lines, or a difference in the rate of transit from the apoptotic to autolytic stage.

The morphological appearance of apoptotic cells precedes a decrease in viability as assessed by nigrosine dye uptake. This is in agreement with studies by Wyllie (in preparation) on MPS-induced cell death in rat thymocytes, where apoptosis precedes uptake of nigrosine by several hours. This suggests that apoptotic cells initially have a still functioning plasma membrane and are therefore capable of metabolic activity; indeed, morphological evidence suggests that apoptotic cells (or fragments) have some functionally intact organelles such as mitochondria (Kerr, Wyllie and Currie, 1972; and see previous section).

The increase in the percentage of autolytic cells (the result of further degradative changes in apoptotic cells) follows the increase in the percentage of nigrosine-stained cells. This reflects the morphological nature of autolysis (Trump and Ginn, 1969) where the cell shows focal dissolution of the plasma membrane, indicating a loss of membrane integrity and therefore leading to entry of nigrosine into the cell.

Thus as a marker of MPS-induced cell death in human lymphoid cells <u>in vitro</u>, nigrosine uptake when compared with morphological changes fairly accurately reflects the cytolethal response at about 48hr/...

48hr after treatment, which was the length of incubation time chosen for routine studies.

PART I

SUMMARY

SECTION 1 The lymphoid cell lines exhibit a marked cytolethal response to MPS only around $10^{-3}$M, a concentration greatly in excess of both physiological and therapeutically attainable glucocorticoid levels *in vivo*: the response was assessed by the ability of live cells to exclude the vital dye, nigrosine, over a 48hr incubation period with MPS. The kinetic studies of the cytolethal response over a 48hr incubation period are consistent with a dose-dependent relationship and the continued presence of MPS is necessary for progression of lethal effects to occur.

SECTION 2 An LM, TEM and SEM study of the morphological aspects associated with the cytolethal response of $BLA_1$ cells to $1.4 \times 10^{-3}$M MPS over a 48hr incubation period, revealed two distinctive morphological changes occurring from 1hr onwards after treatment. One showed contortion and 'blebbing' of the cytoplasm and nucleus resulting in the formation of discrete membrane-bounded fragments of variable size. These contained some apparently normal cytoplasmic organelles, and nuclear fragments which showed pyknosis and nuclear membrane breakdown - in some cases pyknosis preceded cellular and nuclear fragmentation. The other showed 'rounding up' of the cell with loss of cytoplasmic protrusions and long slender surface processes, aggregation of well-preserved cytoplasmic organelles, accompanied by nuclear pyknosis and fragmentation and nuclear membrane breakdown. In both cases many of the features are typical of apoptosis. The subsequent degeneration of cells and fragments not unexpectedly/...

unexpectedly resembled *in vitro* autolysis.

SECTION 3 Over a 48hr incubation period of $BLA_1$ cells with $1.4 \times 10^{-3}$M MPS, the morphological appearance of apoptosis was found to precede a decrease in viability as assessed by nigrosine dye uptake; however the decrease in viability was found to follow the increase in the appearance of autolytic cells. This suggests that nigrosine uptake is a satisfactory 'late' marker in the assessment of the cytolethal response of lymphoid cells to glucocorticoid.

# PART II GROWTH INHIBITORY GLUCOCORTICOID EFFECTS ON HUMAN LYMPHOID CELLS

## SECTION 1

## KINETICS OF THE GROWTH INHIBITORY RESPONSE OF HUMAN LYMPHOID CELLS TO GLUCOCORTICOID

Initially I established the presence of a growth inhibitory - or growth retardation - response in our *in vitro* system. A series of cell lines was tested with MPS within the concentration range $10^{-3}$M - $10^{-7}$M, and the growth inhibitory response was assessed at 144hr (6 days) after treatment with MPS by measurement of TCC and viability.

An experimental system was then devised such that the kinetics of the observed growth inhibitory response could be studied over a more prolonged period of time - this involved feeding cultures every 2 days with fresh growth medium in order to keep them in logarithmic growth phase for at least 2 weeks.

Experiments were also performed to determine whether the continued presence of MPS was necessary for the growth inhibitory response to be maintained.

### MATERIALS AND METHODS

Growth inhibitory test - no feeding

Five tube or flask cultures of $BLA_1$, $GS_1$, RAJI and $EB_4$ cells were set up/...

up in duplicate at a concentration of 0.5 - 1 x $10^5$ total cells/ml by diluting stock cultures with fresh growth medium. Duplicate cultures were immediately treated with 1% (v/v) of stock MPS solution to give concentrations within the range $10^{-7}$M - $10^{-3}$M MPS. Cultures were assessed for viability (nigrosine exclusion) and TCC (Coulter Counter) at 6 days after treatment.

Control cultures were set up in duplicate and treated with 1% (v/v) of sterile distilled water and assessed as for test cultures.

Growth inhibitory test - feeding every 48hr

Six flask cultures of $BLA_1$ and $EB_4$ cells were set up in duplicate at a concentration of 2.5 x $10^5$ total cells/ml by diluting stock cultures with fresh growth medium. Duplicate cultures were immediately treated with 1% (v/v) of stock MPS solution to give concentrations within the range $10^{-8}$M - 0.5 x $10^{-3}$M MPS. Cultures were assessed for viability and TCC every 48hr and fed with fresh growth medium at the same time (as described below) until 16 days after treatment.

Control cultures were set up in duplicate and treated with 1% (v/v) of sterile distilled water and assessed and fed as for test cultures.

Counts and feeding

Every 48hr, 20% (by volume) of 'old' growth medium was removed from each culture without either disturbing or removing any cells which had settled as a layer on the bottom of the flask. The cells were then resuspended by gentle swirling, and 0.5ml removed for assessment of/...

of viability and TCC. The appropriate volume of cell suspension was then removed and discarded and the remaining cells fed with fresh growth medium so that (a) the final volume in each flask was restored to the original volume and (b) the final TCC was restored to 2.5 x $10^5$/ml.

This method of feeding ensured that:

(a) cells were maintained in logarithmic growth phase for the duration of the experiment

(b) all cells, including the more slowly growing, received a regular and substantial supplement of nutrients.

Measurement of doubling time

The doubling time is calculated from the assessment of TCC over one 48hr interval from the following equation:

Doubling time ($t_{DT}$) = $\frac{\log 2}{K}$ where K = growth constant

- see Appendix I (a) (p. 181) for further details on calculation. The mean $t_{DT}$ is calculated from successive 48hr assessments of $t_{DT}$ over the duration of the experiment. In each experiment, the $t_{DT}$ over the first 48hr interval after treating cultures is not included in this value, as the growth inhibitory response is not usually observed until 4 days (96hr) after treatment with MPS.

Cumulative total cell number

This represents the theoretical total cell number in each cell culture after correcting results for all preceding dilutions due to feeding every 48hr, and is calculated from the following equation:

$$[N]_t = \frac{[N]_{t-48}}{n} \times N_t$$

- see/...

- see Appendix I (b) (p. 181) for further details on calculation.

Duration of exposure to growth inhibitory concentration of MPS

Five flask cultures of $EB_4$ cells resuspended in fresh growth medium were set up in duplicate at a concentration of $2.5 \times 10^5$ total cells/ml and immediately treated with 1% (v/v) of stock MPS solution to give a final concentration of $10^{-5}$M MPS. Duplicate cultures were washed x 2 (at 37°C) with fresh growth medium after the desired period of exposure to the glucocorticoid. The cells were resuspended in the original volume of fresh growth medium containing no MPS and then cultured for a further 12 days.

Throughout the duration of the experiment, flasks were fed and assessed for TCC and viability every 48hr as described above.

Five control cultures were set up in duplicate and treated with 1% (v/v) of sterile distilled water. Cells were washed, fed and assessed for TCC and viability as for test cultures.

## RESULTS

### Glucocorticoid sensitivity of lymphoid cell lines

Treatment with MPS for 6 days (144hr) - no feeding of cultures. The log dose-response curves of individual cell lines, as assessed by measurement of TCC and viability at 144hr after treatment with MPS, are shown in figs. 33 and 34 respectively.

With/...

Fig. 33

Effect of methylprednisolone (MPS) on the growth rate of lymphoid cell lines

Cells were treated with various concentrations of MPS ($10^{-7}$ - $10^{-3}$M) (test) or water (control) for 144hr when they were assessed for total cell concentration (TCC). ▲——▲, $GS_1$; O——O, $BLA_1$; □——□, RAJI; ●——●, $EB_4$. Each point represents the mean of duplicate observations from one ($BLA_1$), two (RAJI, $EB_4$) or three ($GS_1$) separate experiments.

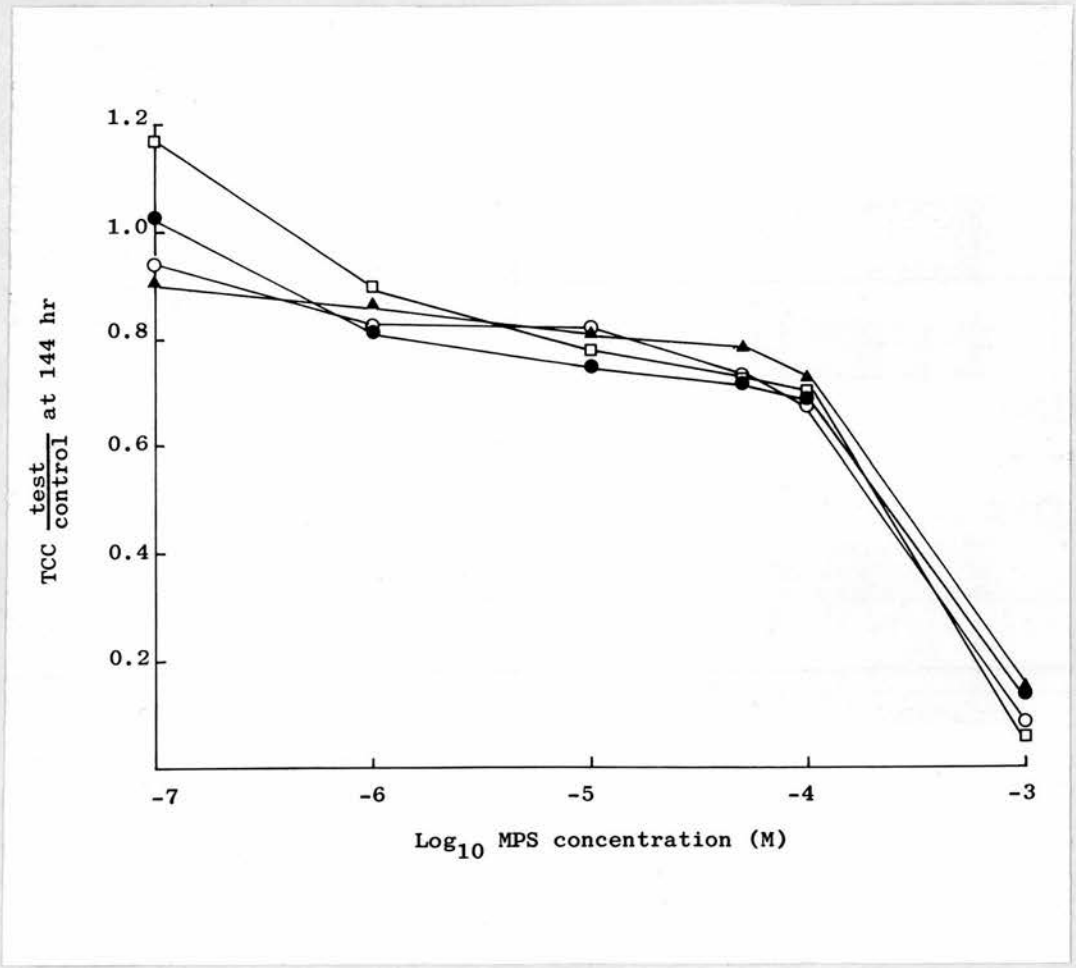


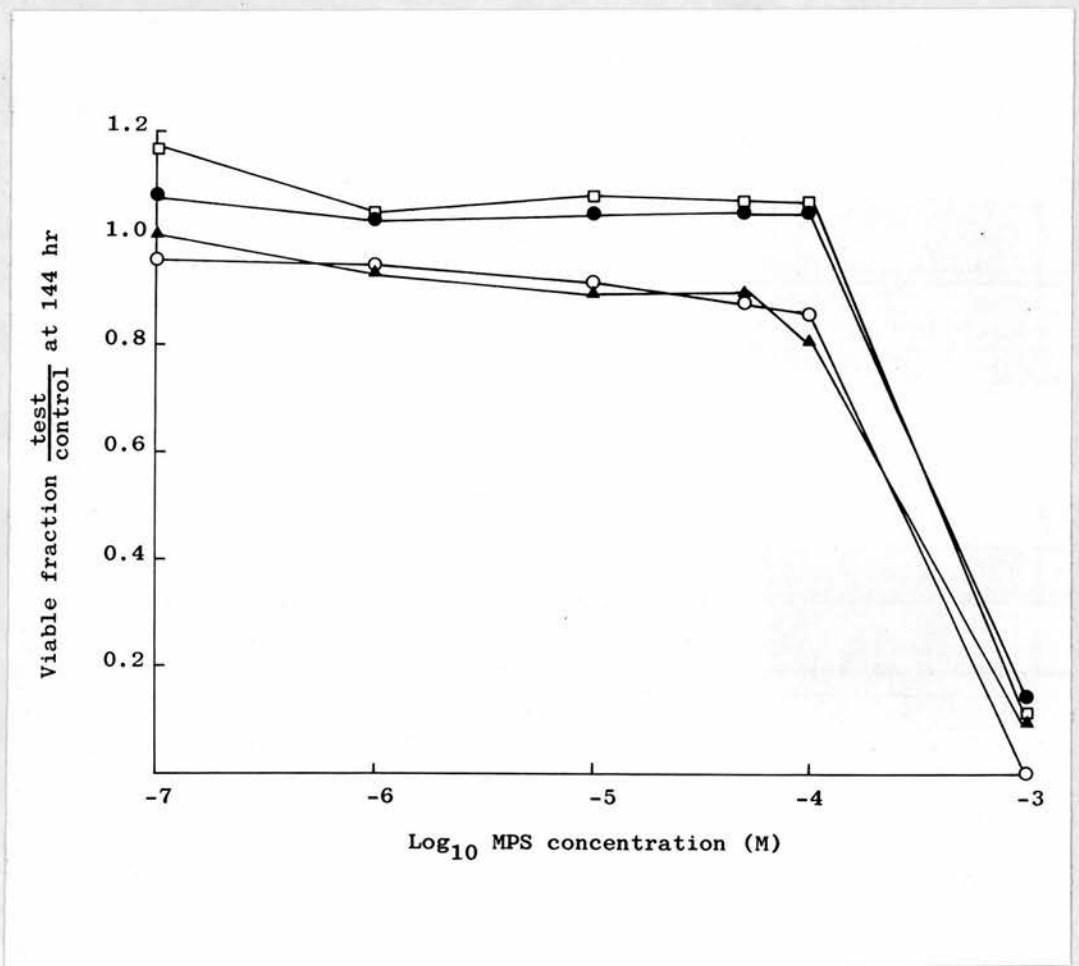
Fig. 34

Effect of methylprednisolone (MPS) on the viability of lymphoid cell lines

Cells were treated with various concentrations of MPS ($10^{-7}$M - $10^{-3}$M) (test) or water (control) for 144hr when they were assessed for viability.

▲——▲, $GS_1$; O——O, $BLA_1$; □——□, RAJI; ●——●, $EB_4$.

Each point represents the mean of duplicate observations from one ($BLA_1$), two (RAJI, $EB_4$) or three ($GS_1$) separate experiments.



With $10^{-3}$M MPS all cell lines show the expected cytolethal response (see Part I of this thesis), with a marked decrease in growth rate (fig. 33) and viability (fig. 34) over the 144hr treatment period.

$EB_4$ and RAJI cells show growth inhibition over the concentration range $10^{-6}$M - $10^{-4}$M MPS (fig. 33) with the TCC of MPS-treated cultures falling to about 70% of control values with $10^{-4}$M MPS - this occurs without any corresponding drop in cell viability (fig. 34).

$GS_1$ and $BLA_1$ cells show growth inhibition over the range $10^{-7}$M - $10^{-4}$M MPS (fig. 33) with TCC values of MPS-treated cultures again falling to about 70% of control values with $10^{-4}$M MPS - however this also occurs with a corresponding small decline in cell viability of MPS-treated cultures over the concentration range, reaching about 81-86% of control values with $10^{-4}$M MPS (fig. 34).

Treatment with MPS for 16 days - cultures fed every 48hr. The growth inhibitory response to MPS was studied further on $EB_4$ and $BLA_1$ cultures over a treatment period of 16 days. To ensure that any growth inhibition observed after MPS treatment was not due to cells reaching plateau growth phase with a limited supply of nutrients, cultures were maintained in logarithmic growth phase for the duration of the experiment by feeding with fresh growth medium every 2 days as described in Materials and Methods (p. 82). Fig. 35 compares the growth rate of fed and unfed (control) cultures, the fed cultures showing a steady increase in the cumulative total cell number over 6 days, whereas the control cultures show a marked retardation of growth by 6 days (144hr).

MPS-/...

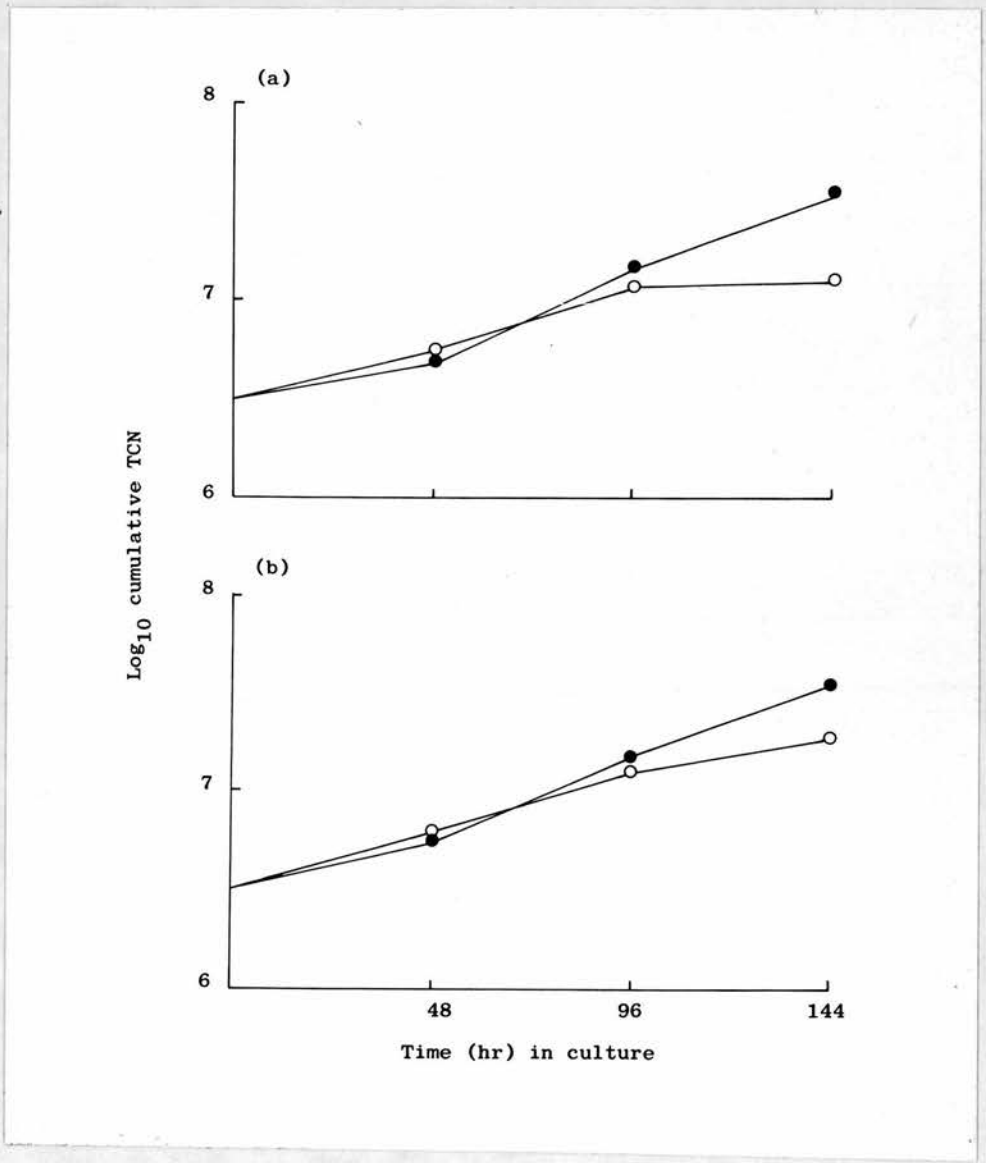
Fig. 35

Comparison of growth rate of fed and unfed (control) cultures of (a) $BLA_1$ and (b) $EB_4$ cells

Cultures were assessed for total cell concentration every 48hr over a period of 6 days. The growth rate is expressed in terms of cumulative total cell number (TCN) (see Appendix I(b), p. 181 for calculation).

●——●, fed every 48hr; ○——○, unfed (control).

Each point represents the mean of duplicate observations from one experiment.



MPS-induced growth inhibition, as represented by an increase in the mean $t_{DT}$ of cells over 16 days treatment with MPS, is observed to be dose-dependent in both $EB_4$ and $BLA_1$ cells within the concentration range $10^{-7}$M - $10^{-4}$M MPS (figs. 36 and 37). For $EB_4$ cells (fig. 36) this response reaches a maximum with around $10^{-5}$M MPS.

Dose-dependent growth inhibition can be similarly represented by a slower rate of increase in the cumulative total cell number in MPS-treated cultures within the concentration range $10^{-7}$M - $10^{-4}$M MPS. Fig. 38 shows such a graph for $EB_4$ cells (typical also for $BLA_1$ cells) and it reveals that the marked growth inhibitory response with $10^{-4}$ - $10^{-6}$M MPS is not evident until after 4 days of treatment; thereafter the response remains stable.

With $0.5 \times 10^{-3}$M MPS-treated $EB_4$ cells (fig. 38) growth inhibition is immediately evident after 2 days of treatment - thereafter a state of no growth is observed, followed by cell loss indicated by a decrease in cumulative cell number. This response is due to a marked decrease in viability of the MPS-treated culture (table 6, p. 93 - final column) which declines steadily from 4 to 6 days after treatment reaching 14% of the control value by 16 days i.e., a cytolethal response is observed.

The viability of cultures treated with $10^{-8}$M - $10^{-4}$M MPS is also shown in table 6 (p. 93) as observed over the 16 day treatment period. $BLA_1$ cells treated with $10^{-8}$M - $10^{-6}$M MPS show no marked viability change throughout the duration of the experiment; $10^{-5}$ - $10^{-4}$M MPS produces a small decrease in viability after 10 - 14 days of treatment/...

Fig. 36

Effect of methylprednisolone (MPS) on the doubling time of $EB_4$ cells

Cultures were treated with various concentrations of MPS ($10^{-8}$M - $10^{-4}$M) or with water (control) for 16 days. Each point represents the mean ± 1S.E. of doubling times of cultures as assessed over successive 48hr feeding intervals from 2-16 days after treatment, in one typical experiment (for calculation see Appendix I(a), p. 181 and Materials and Methods p. 83). The interval between 0 and 2 days was not included as no growth inhibitory effect was yet apparent (see fig. 38).

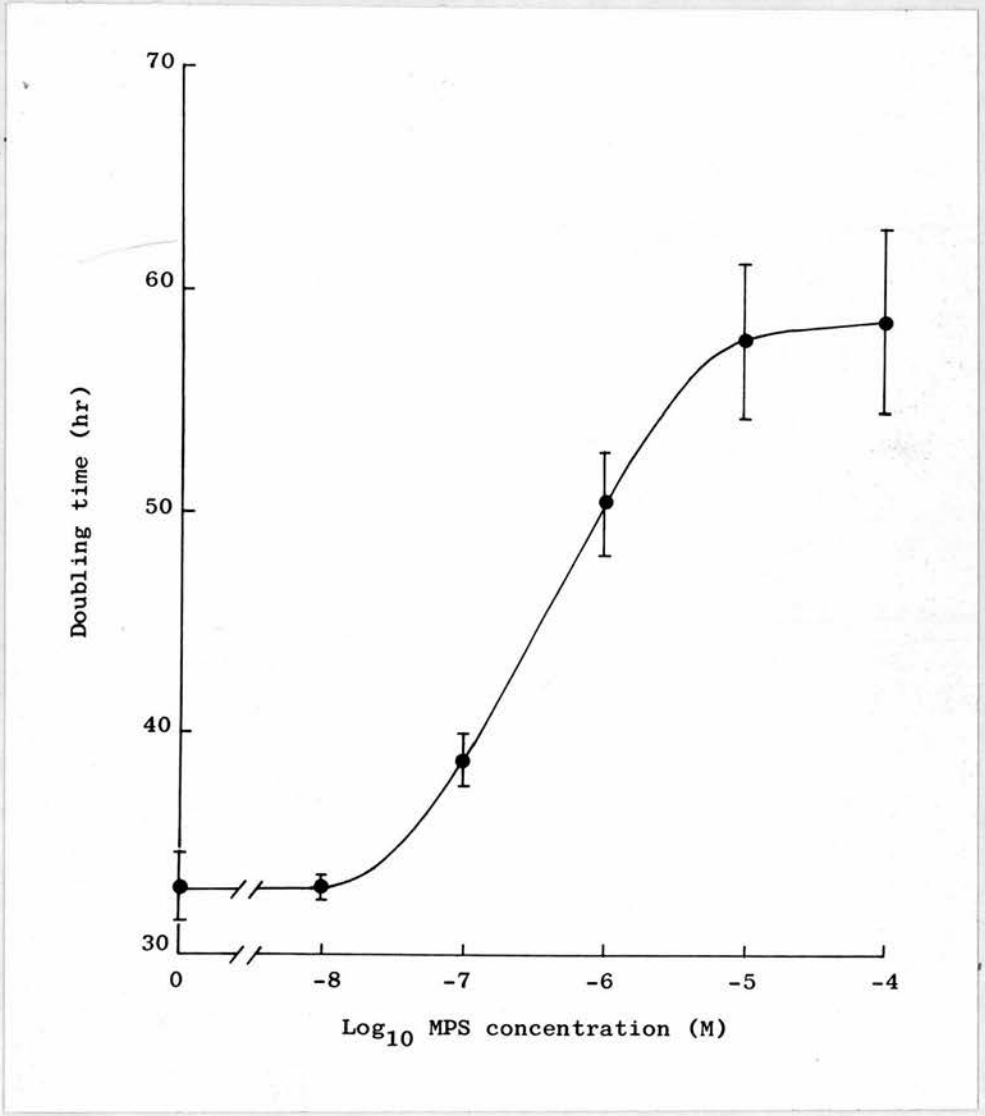


Fig. 37

Effect of methylprednisolone (MPS) on the doubling time of $BLA_1$ cells

Cultures were treated with various concentrations of MPS ($10^{-8}$M - $10^{-4}$M) or with water (control) for 16 days. Each point represents the mean $\pm$ 1S.E. of doubling times of cultures as assessed over successive 48hr feeding intervals from 2-16 days after treatment, in one typical experiment (for calculation see Appendix I(a), p. 181 and Materials and Methods p. 83). The interval between 0 and 2 days was not included as no growth inhibitory effect was yet apparent.

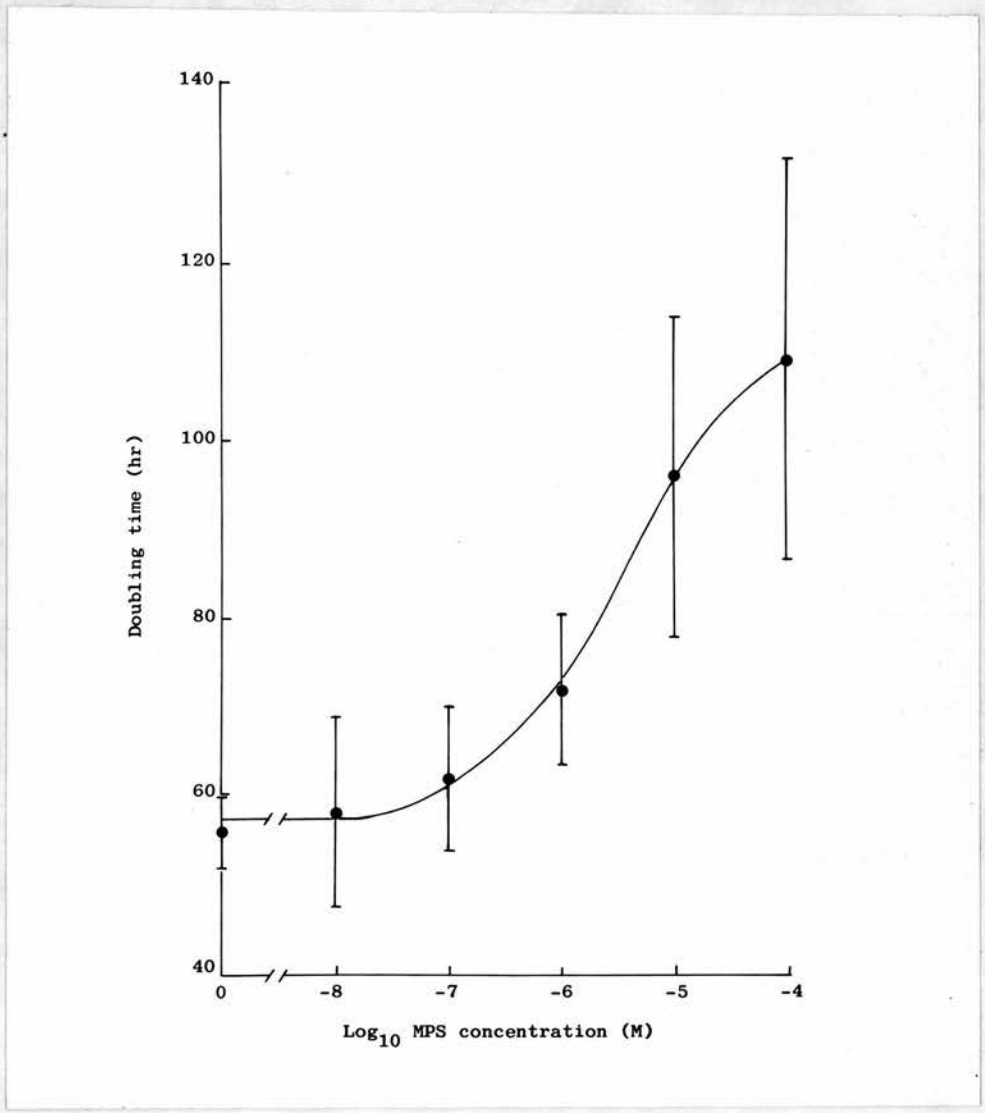


Fig. 38

Effect of methylprednisolone (MPS) on the growth rate of $EB_4$ cells

Cells were incubated with various concentrations of MPS ($10^{-8}$M - 0.5 x $10^{-3}$M) or with water (control) for 16 days. Every 48hr, cultures were assessed for total cell concentration and fed with fresh growth medium. The growth rate is expressed in terms of cumulative total cell number (TCN) (see Appendix I(b), p. 181 for calculation).

■——■, control; □——□, $10^{-8}$M MPS; ▲——▲, $10^{-7}$M MPS; △——△, $10^{-6}$M MPS; ●——●, $10^{-5}$M MPS; ○——○, $10^{-4}$M MPS; ◆——◆, 0.5 x $10^{-3}$M MPS.

Each point represents the mean of duplicate observations from one typical experiment.

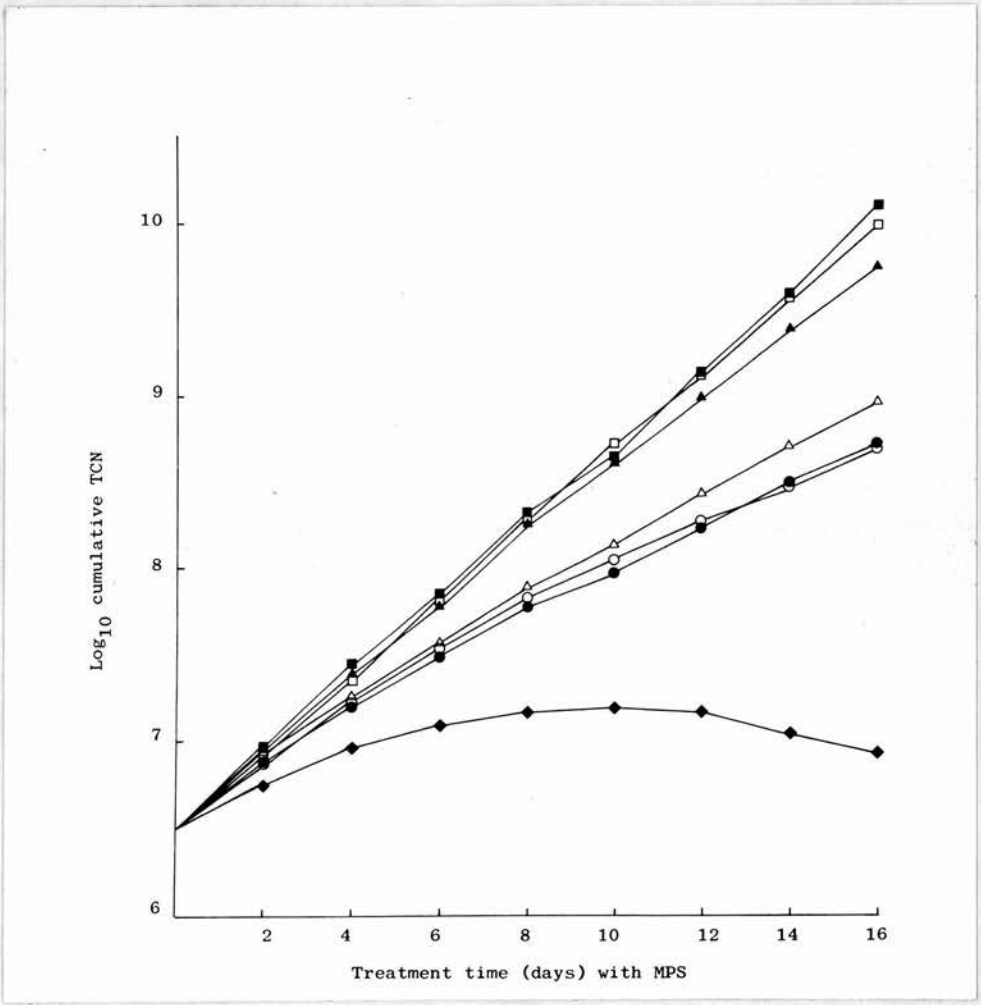


TABLE 6

EFFECT OF METHYLPREDNISOLONE (MPS) ON THE VIABILITY OF $EB_4$ AND $BLA_1$ CELLS

| Cell line | Days in culture | Viable fraction $\frac{\text{test}}{\text{control}}$ using the following concentrations of MPS (M): | | | | | |
|---|---|---|---|---|---|---|---|
| | | $10^{-8}$ | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ | $10^{-4}$ | $0.5 \times 10^{-3}$ |
| $BLA_1$ | 2 | 1.00 | 1.03 | 0.99 | 1.01 | 1.01 | - - - - |
| | 4 | 1.01 | 1.00 | 0.97 | 0.99 | 0.99 | - - - - |
| | 6 | 1.03 | 1.00 | 0.99 | 1.01 | 0.96 | - - - - |
| | 8 | 1.04 | 1.07 | 1.03 | 1.04 | 1.00 | - - - - |
| | 10 | 1.03 | 1.02 | 1.01 | 0.96 | 0.92 | - - - - |
| | 12 | 1.05 | 1.02 | 1.00 | 0.91 | 0.91 | - - - - |
| | 14 | 0.97 | 0.96 | 1.00 | 0.89 | 0.85 | - - - - |
| | 16 | 1.03 | 1.01 | 1.01 | 0.97 | 0.97 | - - - - |
| $EB_4$ | 2 | 0.98 | 0.99 | 0.99 | 1.00 | 1.00 | 0.98 |
| | 4 | 0.98 | 0.99 | 0.98 | 0.97 | 0.97 | 0.94 |
| | 6 | 0.99 | 0.99 | 0.95 | 0.97 | 0.97 | 0.85 |
| | 8 | 1.00 | 0.97 | 0.98 | 0.92 | 0.93 | 0.68 |
| | 10 | 0.98 | 1.01 | 0.97 | 0.86 | 0.93 | 0.60 |
| | 12 | 1.01 | 1.01 | 0.92 | 0.87 | 0.89 | 0.28 |
| | 14 | 0.99 | 0.99 | 0.95 | 0.89 | 0.91 | 0.15 |
| | 16 | 0.98 | 0.98 | 0.93 | 0.88 | 0.86 | 0.14 |

Cells were treated with various concentrations of MPS ($10^{-8}$M - $0.5 \times 10^{-3}$M) (test) or water (control) for 16 days. Every 48hr, cultures were assessed for viability and fed with fresh growth medium. - - - -, no results available.

Each value represents the mean of duplicate observations from one typical experiment.

treatment - at most 15% below the control value. Similar but somewhat more pronounced effects are noted in $EB_4$ cells: there is a downward trend in viability - at its maximum about 14% below control values, in cells treated with $10^{-5}$M or $10^{-4}$M MPS for 10 - 16 days.

Effect of removal of $10^{-5}$M MPS on growth inhibitory response after various periods of treatment with MPS

The duration of the glucocorticoid pulse to which $EB_4$ cells were exposed was varied by washing cultures free of a growth inhibitory concentration of MPS - $10^{-5}$M - at 4, 6, 10 and 14 days after treatment, time intervals known to show a growth inhibitory response from previous experiments; cultures were then grown for a further 12 days after washing. Throughout the duration of the experiments, cultures were fed with fresh growth medium every 2 days (see Materials and Methods, p. 82).

Table 7 (p. 95) shows the effect of washing on the $t_{DT}$'s of both MPS-treated and control cultures. For each period of incubation, the $t_{DT}$'s of pre-wash and post-wash control cultures remains within approximately the same range (36.9-39.0hr and 33.6-37.5hr respectively) which suggests that the washing procedure and handling of cells has no detrimental effect on the growth rate of $EB_4$ cultures. During MPS treatment, the characteristic long $t_{DT}$ is observed (ranging from 47.8-58.1hr), and for each period of treatment with MPS the $t_{DT}$ returns to control levels after washing free of MPS. The $t_{DT}$'s for control and MPS-treated cultures grown continuously for 26 days without washing are within the range for control and MPS-treated cultures incubated for 4, 6, 10 and 14 days and confirms that the method/...

TABLE 7

EFFECT OF REMOVAL OF $10^{-5}$M METHYLPREDNISOLONE (MPS) ON DOUBLING TIME OF $EB_4$ CELLS

| Period of treatment (days) | Doubling time (hr) ± 1S.E. for cultures: | | | |
|---|---|---|---|---|
| | Control | | MPS-treated | |
| | Pre-wash | Post-wash | Pre-wash | Post-wash |
| 4 | 36.9 ± 2.3 | 37.5 ± 1.7 | 58.1 ± 9.0 | 37.1 ± 1.1 |
| 6 | 37.9 ± 1.0 | 36.8 ± 1.7 | 57.3 ± 2.8 | 37.2 ± 1.4 |
| 10 | 39.0 ± 2.6 | 35.2 ± 1.8 | 56.9 ± 2.5 | 39.4 ± 1.8 |
| 14 | 38.0 ± 1.3 | 33.6 ± 2.6 | 47.8 ± 1.1 | 38.8 ± 2.2 |
| 26 (continuous) | 39.0 ± 1.1 | - - - | 55.0 ± 1.6 | - - - |

After various periods of treatment with MPS or water (control) (as shown), cultures were washed free of treatment, resuspended in fresh growth medium and cultured for a further 12 days. Cultures were assessed for total cell concentration every 48hr before and after washing and fed with fresh growth medium every 48hr.

Doubling times (hr) ± 1S.E. are assessed by meaning values over successive 48hr intervals from 2 separate experiments for periods equivalent to those shown on fig. 39 (p. 97) - for calculation see Appendix I(a), p. 181 . (The 48hr interval after initial addition of MPS is not included as no growth inhibitory effect was yet apparent.)

method of feeding employed allows cells to remain in logarithmic growth phase for a prolonged period of time.

Fig. 39 shows the effect of washing cultures free of $10^{-5}$M MPS on the cumulative total cell number after 10 days treatment with MPS - this graph typifies the effect seen also with 4, 6, and 14 days treatment. As seen in previous experiments, the growth inhibitory effect is not observed until 4 days after treatment with MPS. After washing at 10 days, the growth rate of the MPS-treated culture then returns to the faster growth rate observed in the control culture.

The viabilities of MPS-treated cultures both before and after washing free of $10^{-5}$M MPS are shown in table 8,/ p. 99. Where a small decrease in viability precedes washing (with 6, 10 and 14 days incubation), a slow increase occurs again after washing free of MPS. The viability of cultures treated continuously with MPS, which drops to $\leq 90\%$ of the control value after 10 days incubation, shows a small increase again after 20 days.

## COMMENT

Initial experiments on a range of lymphoid cell lines, when assessing for TCC at 6 days after treatment, suggested the existence of a growth inhibitory response to lower concentrations of MPS ($10^{-7}$M - $10^{-4}$M) than that required to induce a cytolethal response ($10^{-3}$M).

The MPS-induced growth inhibitory response was then studied in more/...

Fig. 39

Effect of removal of $10^{-5}$M methylprednisolone (MPS) on growth rate and doubling time of $EB_4$ cells

After 10 days treatment with $10^{-5}$M MPS or water (control), cultures were washed free of treatment, resuspended in fresh growth medium and cultured for a further 12 days. Cultures were assessed for total cell concentration every 48hr before and after washing and fed with fresh growth medium every 48hr. The growth rate is expressed in terms of cumulative total cell number (TCN) (see Appendix I(b), p. 181 for calculation). O——O, control culture; ●——●, $10^{-5}$M MPS-treated culture.

Each point represents the mean of duplicate observations from 2 separate experiments. Doubling times (DT) (hr) ± 1S.E. are assessed by meaning values over successive 48hr intervals for the periods shown on the graph (for calculations see Appendix I(a), p. 181 ).

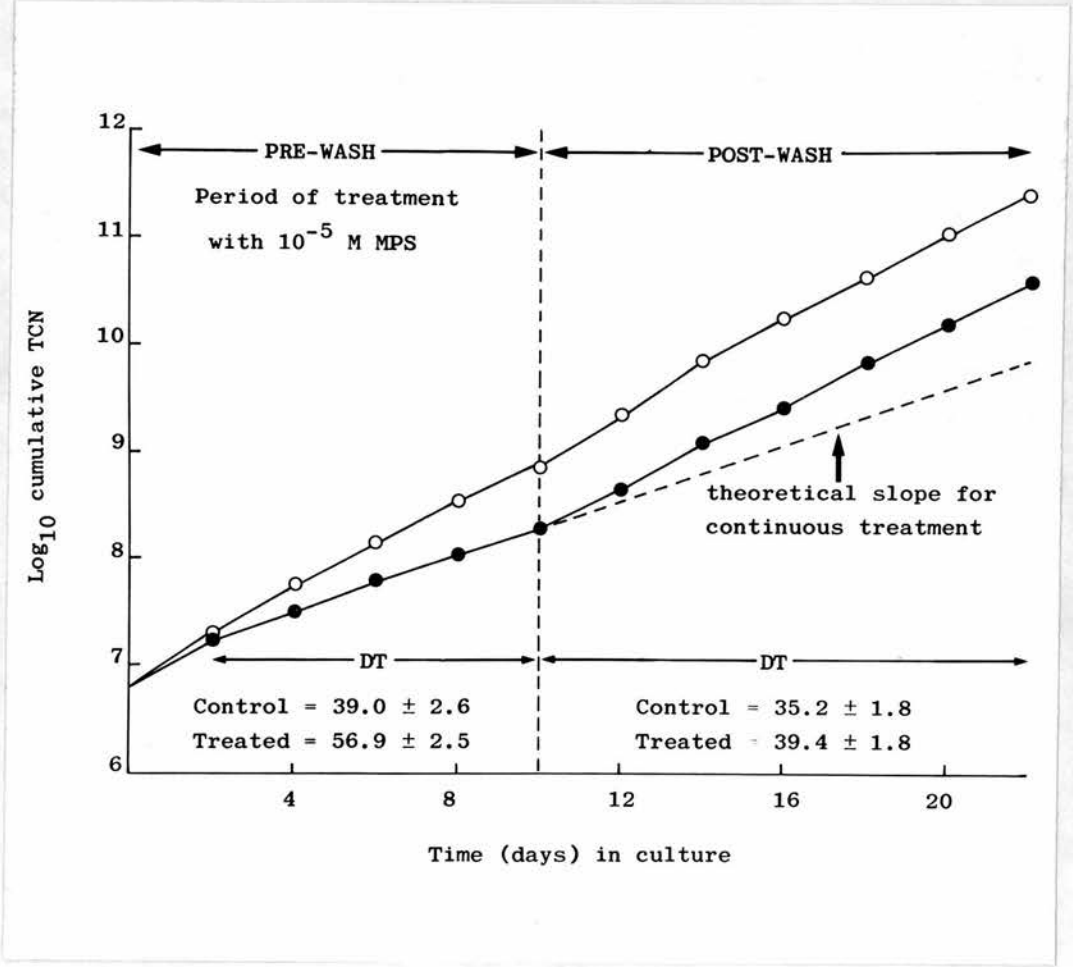


TABLE 8

After various periods of treatment with MPS (test) or water (control) (as shown), cultures were washed free of treatment, resuspended in fresh growth medium and cultured for a further 12 days. Cultures were assessed for viability every 48hr before and after washing and fed with fresh growth medium every 48hr. Underlining denotes the time at which cultures were washed; ....., no further assessments taken after 12 days post-wash. Each value represents the mean $\pm$ 1S.E. of duplicate observations from 2 separate experiments.

TABLE 8 EFFECT OF REMOVAL OF $10^{-5}$M METHYLPREDNISOLONE (MPS) ON VIABILITY OF $EB_4$ CELLS

| Time (days) in culture | Viable fraction $\frac{\text{test}}{\text{control}}$ ± 1S.E. for $EB_4$ cells treated for the following period of time (days) before washing: | | | | |
|---|---|---|---|---|---|
| | 4 | 6 | 10 | 14 | 26(continuous) |
| 0 | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 |
| 2 | 1.02 ± 0.01 | 0.98 ± 0.01 | 1.01 ± 0.01 | 0.99 ± 0.01 | 0.99 ± 0.00 |
| 4 | 0.99 ± 0.02 | 1.00 ± 0.01 | 0.99 ± 0.01 | 0.97 ± 0.02 | 0.99 ± 0.01 |
| 6 | 0.98 ± 0.01 | 0.94 ± 0.02 | 0.97 ± 0.01 | 0.98 ± 0.01 | 0.94 ± 0.02 |
| 8 | 0.98 ± 0.01 | 0.89 ± 0.01 | 0.93 ± 0.02 | 0.95 ± 0.01 | 0.95 ± 0.02 |
| 10 | 0.99 ± 0.01 | 0.93 ± 0.01 | 0.88 ± 0.02 | 0.94 ± 0.01 | 0.90 ± 0.02 |
| 12 | 1.00 ± 0.01 | 0.99 ± 0.01 | 0.88 ± 0.03 | 0.90 ± 0.03 | 0.85 ± 0.02 |
| 14 | 0.96 ± 0.01 | 0.99 ± 0.02 | 0.92 ± 0.01 | 0.94 ± 0.02 | 0.90 ± 0.01 |
| 16 | 0.97 ± 0.01 | 0.98 ± 0.01 | 0.93 ± 0.03 | 0.93 ± 0.01 | 0.88 ± 0.02 |
| 18 | ..... | 0.99 ± 0.01 | 0.93 ± 0.01 | 0.94 ± 0.01 | 0.89 ± 0.01 |
| 20 | ..... | ..... | 0.95 ± 0.02 | 0.96 ± 0.01 | 0.94 ± 0.03 |
| 22 | ..... | ..... | 0.96 ± 0.02 | 0.96 ± 0.01 | 0.94 ± 0.01 |
| 24 | ..... | ..... | ..... | 0.99 ± 0.02 | 0.98 ± 0.01 |
| 26 | ..... | ..... | ..... | 0.99 ± 0.01 | 0.94 ± 0.01 |

more detail on cultures kept in logarithmic growth phase for at least 2 weeks by feeding every 2 days with fresh growth medium. $EB_4$ and $BLA_1$ cells both showed a dose-dependent growth inhibitory response within the concentration range $10^{-7}$M - $10^{-4}$M MPS as shown by either an increase in the population $t_{DT}$ or a slower rate of increase in the cumulative total cell number over 16 days treatment with MPS. The continued presence of MPS was found to be necessary for the growth inhibitory response to be maintained. For $EB_4$ cells, the maximum growth inhibitory response was reached with $10^{-5}$M MPS; $0.5 \times 10^{-3}$M MPS elicited the expected cytolethal response with massive cell death occurring. For $BLA_1$ cells, the MPS concentration range studied was not sufficient to give a maximum growth inhibitory response.

A similar growth inhibitory effect has previously been shown in vitro on cortisone-treated human lymphoid cells by Nilsson (1971) and on hydrocortisone-treated HeLa cells by Kollmorgen (1969) who also found the glucocorticoid effect to be reversible.

A small decrease in viability occurred with $EB_4$ and $BLA_1$ cells (though less markedly for the latter) within the concentration range $10^{-6}$ - $10^{-4}$M MPS, though the relationship with the observed growth inhibitory response is not clear. Theoretically, growth inhibition could be due to:

(a) an increase in cell deletion by death

(b) a decrease in cell production with no loss of viability which could involve an increase in cell cycle time or decrease in growth fraction.

However/...

However results here suggest that although cell death is involved to a small extent it can only be an indirect consequence of (b) above as:

(i) reduction in viability was first observed several days after establishment of growth inhibition (table 6, p. 93)

(ii) after 20 days of continuous MPS treatment the viability increased again (table 8, p. 99), although growth inhibition remained stable (table 7, p. 95).

This finding is in marked contrast to recent work by Norman and Thompson (1977) who showed that glucocorticoid-induced growth inhibition in an uncloned human lymphoid cell line (CEM) is due directly to the death of a sub-population of cells with a sensitive phenotype. Continuing proliferation of the sub-population of resistant phenotype then restores the overall growth rate of the population to that of the control.

In contrast to the glucocorticoid-induced cytolethal response in human lymphoid cell lines, the growth inhibitory response occurs with concentrations of glucocorticoid equivalent to the therapeutic doses employed in clinical practice. It is possible then that the growth inhibitory response plays an important rôle in the regression of lymphoid tumours after glucocorticoid therapy.

The correlation between cytoplasmic glucocorticoid receptors in lymphoid cell lines and the MPS-induced growth inhibitory effect is discussed later (see General Discussion, p. 157).

## SECTION 2

## MORPHOLOGY OF THE GROWTH INHIBITORY RESPONSE OF HUMAN LYMPHOID CELLS TO GLUCOCORTICOID

In the previous section, it was shown that cell death is involved in the MPS-induced growth inhibitory response to a small extent (as observed by nigrosine exclusion). To my knowledge, little information exists as to the morphological changes involved in growth inhibition in human lymphoid cells, although Werthamer and Amaral (1975) have shown changes associated with sub-lethal injury after treatment of normal human lymphocytes with cortisol. This section therefore studies the morphology of $EB_4$ cells during growth inhibition induced by $10^{-5}$M MPS as observed by both LM and TEM.

Combined with this study, the kinetics of observed LM morphological changes are compared with the kinetics of nigrosine uptake over the period of treatment of $EB_4$ cells with $10^{-5}$M MPS.

### MATERIALS AND METHODS

#### Kinetics and morphological studies

Duplicate flask cultures of $EB_4$ cells were set up at a concentration of $2.5 \times 10^5$ total cells/ml by diluting stock cultures with fresh growth medium and then were immediately treated with 1% (v/v) of stock MPS solution to give a final concentration of $10^{-5}$M MPS.

Control/...

Control cultures were treated with 1% (v/v) of sterile distilled water. Cultures were fed and assessed for viability and TCC every 48hr for the duration of the experiment (as described in Materials and Methods, Part II, Section 1, p. 82). For morphological studies, samples were removed at appropriate time intervals and prepared for both LM (smears) and TEM (as described previously in Materials and Methods, Part I, Section 2, p. 41).

## RESULTS

### Control $EB_4$ cells

The typical morphology of $EB_4$ cells, as observed both in smears and ultrastructurally, is illustrated in figs. 40 and 41 respectively. Features are essentially the same as for $BLA_1$ cells (cf. figs. 13 and 14, pp. 45 and 46), except that the surfaces of $EB_4$ cells show more numerous blunt cytoplasmic protrusions and lack long slender surface processes.

### Treated $EB_4$ cells

LM. The majority of the population of $EB_4$ cells as seen on smears appear morphologically normal even at 16 days after treatment with $10^{-5}$M MPS (fig. 42). However from 4 days onwards after treatment, a very small percentage of cells show morphological changes typical of apoptosis and autolysis (fig. 43) as previously described for $BLA_1$ cells after treatment with $1.4 \times 10^{-3}$M MPS (see Part I, Section 2); coincidently there is an increase in the number of cells taking up nigrosine (fig. 43).

TEM/..

Fig. 40

Control $EB_4$ cells

The cell surface shows cytoplasmic protrusions (P) and the cytoplasm contains many clear vacuoles. The cells have a high nuclear to cytoplasmic ratio, with the nucleus sometimes showing a prominent nucleolus (NU). G. x 580.

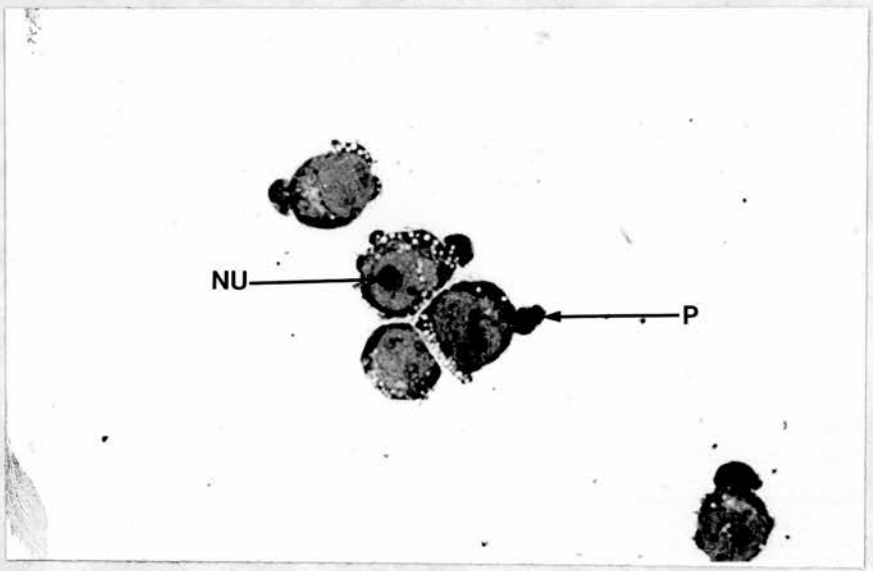


Fig. 41

TEM of control $EB_4$ cells

The cytoplasm is rich in polysomes (P) with scanty profiles of rough endoplasmic reticulum (RER), mitochondria with plate-like cristae (M), and golgi apparatus (G); although often seen, no lipid droplets are present in this cell. The nucleus (N) shows diffusely dispersed chromatin with scanty aggregates of heterochromatin along the nuclear membrane. UALC. x 10,400.

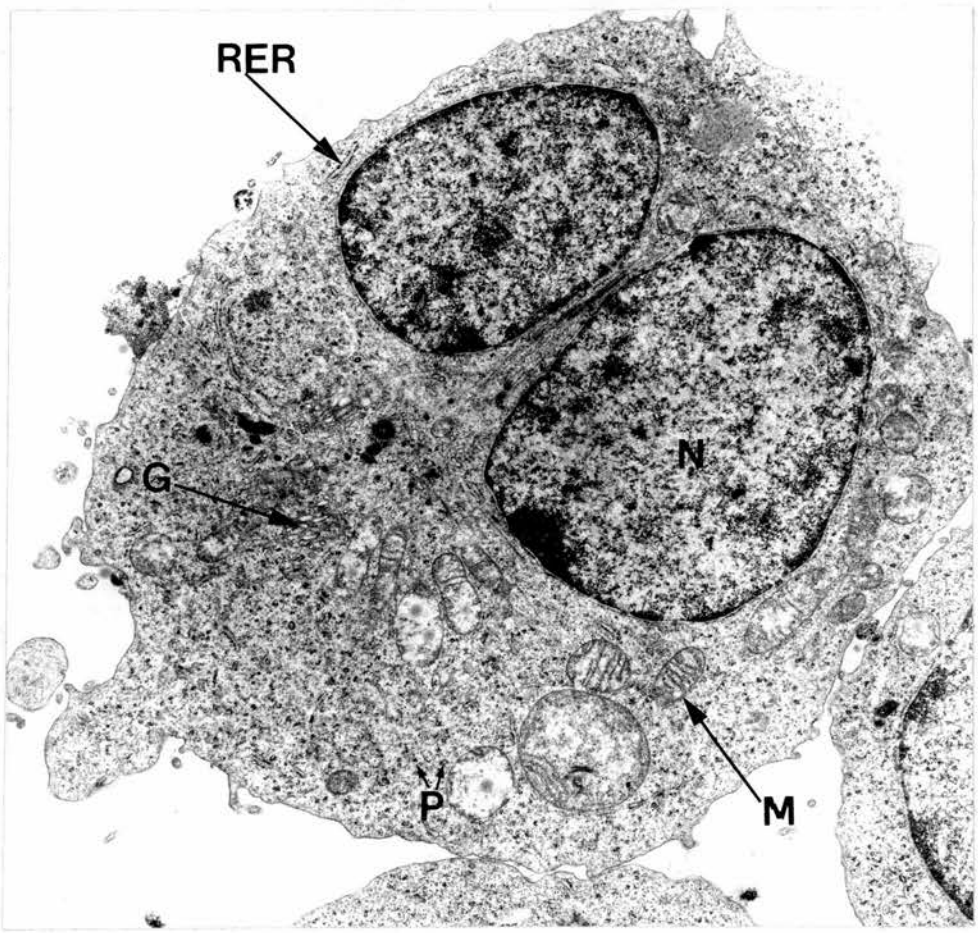


Fig. 42

$EB_4$ cells 16 days after treatment with $10^{-5}$M methylprednisolone
The cells show the same morphological features as for control cells. G. x 580.

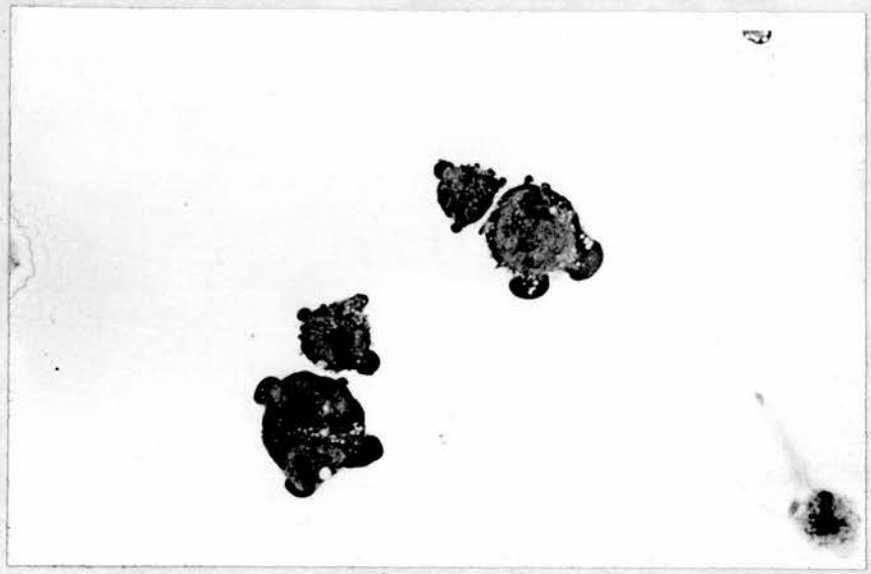
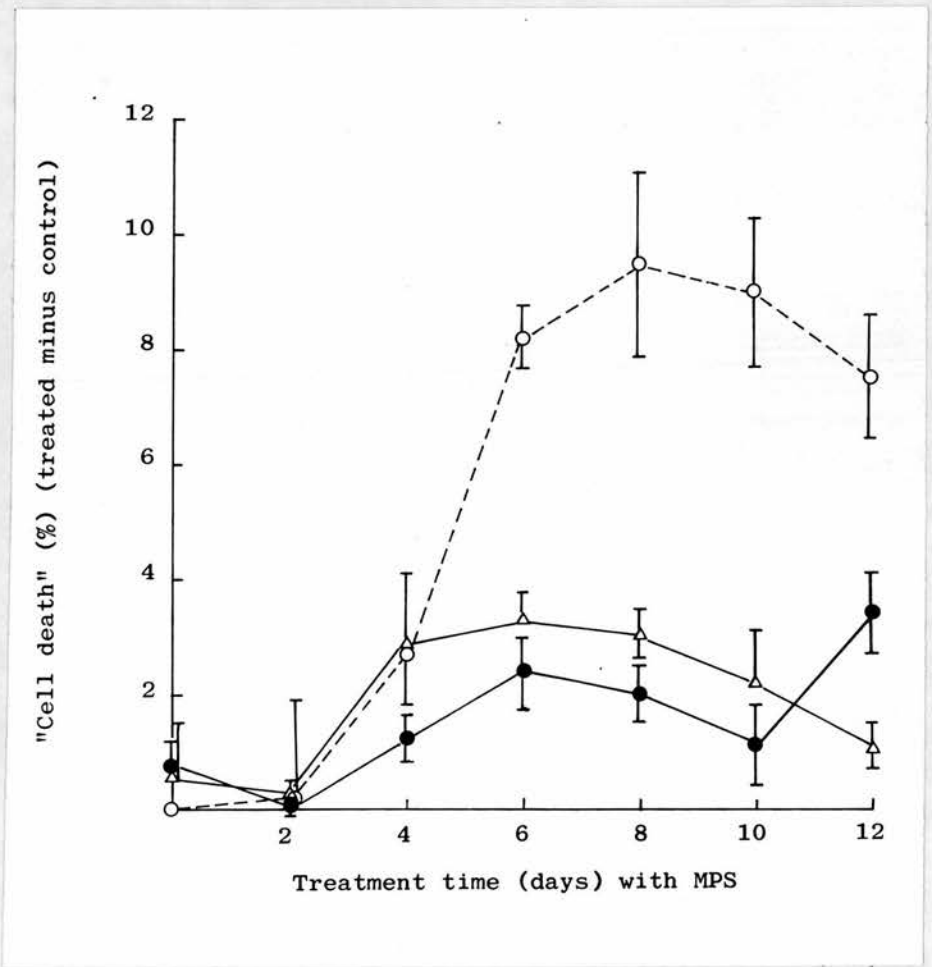


Fig. 43

Kinetics of the morphological changes associated with the growth inhibitory response of $EB_4$ cells to $10^{-5}$M methylprednisolone (MPS)

Cultures were incubated with $10^{-5}$M MPS (treated) or water (control) for 12 days and assessed every 48hr for: morphological change, as observed from smear preparations (1000 cells scored per smear); and viability, as measured by the ability of live cells to exclude nigrosine (200 cells scored per observation) ●——●, apoptotic cells, △——△, autolytic cells, O- - - -O, nigrosine-stained cells.

Each point represents the mean ± 1S.E. of treated minus control values for duplicate observations from 2 separate experiments. Error bars are not included where overlapping occurs.



TEM. At 4 days after treatment with $10^{-5}$M MPS, evidence of ultrastructural damage, other than apoptotic and autolytic changes, is present in some cells - gross mitochondrial changes are seen with varying degrees of disorganisation of mitochondrial cristae and formation of myelin figures with lamellar configuration within the mitochondria (fig. 44); not all mitochondria within one cell show these figures.

## COMMENT

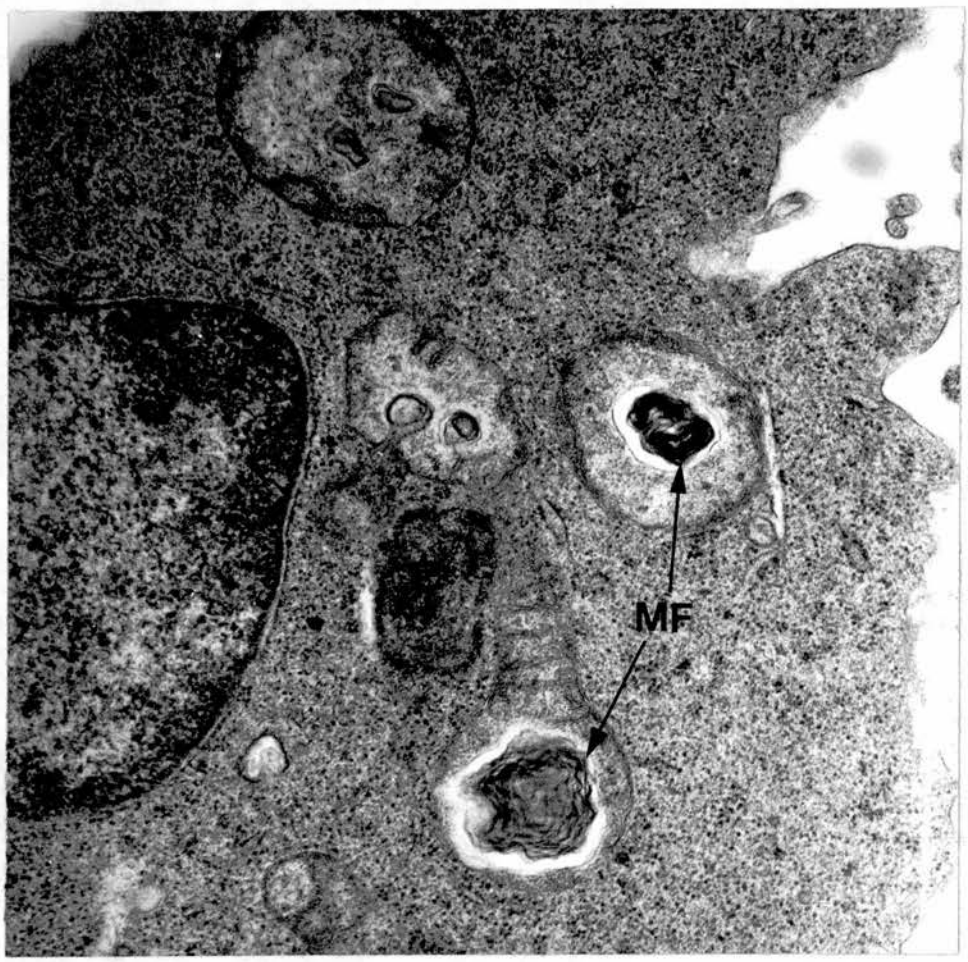
Evidence of sub-lethal MPS damage was not observed in smears even after 16 days treatment with $10^{-5}$M MPS. However with TEM, gross mitochondrial alterations were seen after 4 days treatment which included disorganisation of cristae and formation of myelin figures. These changes are generally regarded as degenerative and indicative of an ongoing autophagic process (Trump and Arstila, 1971) and are similar to the sub-lethal changes reported by Werthamer and Amaral (1975) after treatment of normal human lymphocytes in vitro with cortisol.

The presence in smears of a small increase in the number of apoptotic and autolytic cells after 4 days treatment suggests the presence of a small sub-population of $EB_4$ cells which show a cytolethal response to $10^{-5}$M MPS, a concentration 100-fold less than that required to induce a cytolethal response within the entire population ($10^{-3}$M). These could represent the subsequent death of sub-lethally damaged cells or alternatively could represent death of a/...

Fig. 44

TEM of $EB_4$ cell 4 days after treatment with $10^{-5}$M methylprednisolone

The mitochondria show disorganisation of cristae and the formation of myelin figures (MF). UALC. x 39,000.



a small population of cells sensitive to the lethal effect of $10^{-5}$M MPS. These alternatives could have been distinguished in TEM studies by investigating the presence or absence of such mitochondrial changes in apoptotic cells - unfortunately the percentage of apoptotic cells in treated cultures was too small for this to be possible.

A correlation between the observed morphological changes and biochemical investigations is discussed later (see General Discussion, p. 159).

SECTION 3

# QUANTITATIVE AUTORADIOGRAPHIC ANALYSIS OF THE GROWTH INHIBITION INDUCED IN HUMAN LYMPHOID CELLS BY GLUCOCORTICOID

The increase in population $t_{DT}$ observed in lymphoid cell lines treated with a growth inhibitory concentration of MPS could be due to the following effects on cell kinetic parameters:

(a) an increase in the cell cycle time

(b) a decrease in the growth fraction

(c) an increase in the rate of cell death

(d) a combination of (a), (b) and (c).

In previous population kinetic studies on human lymphoid cells and on other cells both *in vitro* and *in vivo*, it has been shown that the glucocorticoid-induced growth inhibitory response occurs due to a blockage of the transition of cells from $G_1$ into S. However, evidence is conflicting as to whether the blocked cells maintain the capacity to return to cycle giving the appearance of a prolonged $t_{G1}$ (Kollmorgen, 1969; Braunschweiger, Stragand and Schiffer, 1978) or whether the cells are irreversibly committed to die (Norman, Harmon and Thompson, 1978).

From morphological evidence in the previous section it would appear that cell death plays a rôle in glucocorticoid-induced growth inhibition/...

inhibition in $EB_4$ cells; this could possibly be due to death of cells blocked in $G_1$. As a better understanding of such mechanisms of glucocorticoid action would be valuable in designing more effective chemotherapeutic régimes *in vivo* which combine glucocorticoids with other cell cycle active drugs, I have therefore studied the growth inhibitory response of $EB_4$ cells to $10^{-5}$M MPS by analysis of the following kinetic parameters:

(a) number of cells in S-phase (tritiated-thymidine ($^3$H-TdR) pulse-labelling index)

(b) mitotic index

(c) length of the cell cycle time and duration of M, $G_1$, $G_2$ and S phases of the cell cycle (percentage of labelled mitoses technique - pulse-label with $^3$H-TdR; and $^3$H-TdR continuous-labelling technique)

(d) growth fraction (percentage of labelled mitoses technique - pulse-label with $^3$H-TdR; and $^3$H-TdR continuous-labelling index).

$^3$H-TdR is widely used for autoradiographic studies in cell population kinetics; it is a radioactive precursor entirely specific to DNA (Quastler and Sherman, 1959) and the short range of β-ray emission from $^3$H decay allows precise sub-cellular localisation of incorporated nucleoside.

However it has been shown that if used at too high a concentration, the radioactive precursor can cause observable radiation damage in cells which have incorporated it. Such damage includes growth inhibition due mainly to a $G_2$ + M block (Ehmann *et al*, 1975) and may also include mutations, chromosome aberrations and cell death (Bedford *et al*, 1975) and strand breaks in the DNA molecule (Cleaver, Thomas/...

Thomas and Burki, 1972). The presence of excess thymidine itself can cause a reversible inhibition of DNA synthesis and indeed as such is used as a technique for synchronising cell cultures (Mitchison, 1971) - this inhibition arises due to overloading the complex metabolic pool with exogenous thymidine.

Thus in this experimental system it was necessary to carry out preliminary experiments to ensure that the concentration of $^{3}$H-TdR used for either pulse-labelling or continuous-labelling was not itself sufficient to perturb the growth rate of the $EB_4$ cells.

## MATERIALS AND METHODS

### $^{3}$H-TdR

An aqueous solution of 6 - $^{3}$H-TdR, specific activity 21.5 Ci/mM, was obtained from Radiochemicals, Amersham.

### Pulse-labelling with $^{3}$H-TdR

For the preliminary experiment, five flasks of $EB_4$ cells were set up in duplicate at 1 - 2 x $10^5$ cells/ml and duplicate flasks were treated with 1% (v/v) of stock $^{3}$H-TdR solutions to give concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 $\mu$Ci/ml. The cells were incubated with $^{3}$H-TdR for 30 min. at 37°C and then washed free of unbound $^{3}$H-TdR three times with ice-cold Hepes-buffered Hanks solution (Hanks Balanced Salt Solution, Gibco-Biocult; 20mM Hepes buffer, Gibco-Biocult). Cells were finally resuspended in fresh growth medium at the original concentration and allowed to continue growth for 6 days, being fed with fresh growth medium and assessed for TCC and/...

and viability every 2 days (for further details see Appendix II, p. 182). Duplicate control cultures were treated with 1% (v/v) of sterile distilled water for 30 min. at 37°C, then washed, resuspended in fresh growth medium, fed and assessed as for $^{3}$H-TdR-treated cultures.

Continuous-labelling with $^{3}$H-TdR

For the preliminary experiment, four flasks of both control (water) and $10^{-5}$M MPS-treated $EB_4$ cells were set up in duplicate at 2.5 x $10^5$ cells/ml and after 4 days incubation duplicate cultures were treated continuously for 10 days with 1% (v/v) of stock $^{3}$H-TdR solutions to give concentrations of 0.1, 0.05, 0.01 and 0.005 $\mu$Ci/ml. Flasks were assessed for viability and TCC and fed every 2 days (see Materials and Methods Part II, Section 1, p. 82).

$^{3}$H-TdR pulse-labelling index (LI) and mitotic index (MI)

Flasks of both $10^{-5}$M MPS-treated and control (water) $EB_4$ cells were set up in duplicate at 2.5 x $10^5$ cells/ml, and were fed and assessed for TCC and viability every 48hr over a period of 12 days (see Materials and Methods Part II, Section 1, p. 82). At selected time intervals, samples were removed from all flasks and (a) smeared and stained with Giemsa (see Materials and Methods Part I, Section 2, p. 41) for measurement of MI, or (b) pulse-labelled with 1.0 $\mu$Ci/ml $^{3}$H-TdR for 30 min. at 37°C and smears of cells then prepared for autoradiography for measurement of $^{3}$H-TdR LI.

Percentage labelled mitoses technique (PLM)-pulse-label with $^{3}$H-TdR

Flasks of $10^{-5}$M MPS-treated and control (water) $EB_4$ cells were set up/...

up in duplicate at 2.5 x $10^5$ cells/ml, and were fed and assessed for TCC and viability every 48hr (see Materials and Methods Part II, Section 1, p. 82). After 6 days incubation, control and $10^{-5}$M MPS-treated cultures were pulse-labelled with 0.1 µCi/ml $^3$H-TdR for 30 min. at 37°C and washed and resuspended in fresh growth medium as described above under 'Pulse-labelling with $^3$H-TdR' (treatment with water and $10^{-5}$M MPS was maintained). After pulse-labelling, samples were then removed from treated and control cultures at 4hr intervals up to 96hr, and smears of cells prepared for autoradiography.

$^3$H-TdR continuous-LI and PLM curve

Flasks of $10^{-5}$M MPS-treated and control (water) $EB_4$ cells were set up in duplicate at 2.5 x $10^5$ cells/ml and fed and assessed for TCC and viability every 48hr (see Materials and Methods Part II, Section 1, p. 82). After 6 days incubation, all cultures were continuously exposed to 0.01 µCi/ml $^3$H-TdR. Samples were removed at selected time intervals after addition of $^3$H-TdR and smears of cells prepared for autoradiography.

Autoradiography

Smears of cells were prepared on glass slides, air-dried and fixed in 95% methanol for 15 min. Slides were dipped in liquid emulsion (Ilford K5, diluted 2:1 with 1% glycerol) at 43°C in the dark for various periods of time: 5 days for 1 µCi/ml $^3$H-TdR; 5 weeks for 0.1 µCi/ml $^3$H-TdR; 6½ weeks for 0.01 µCi/ml $^3$H-TdR. The emulsion was then developed in Kodak D19 developer for 3½ min., rinsed and fixed in Kodak metafix for 10 min. Slides were then stained with Giemsa. A 'cold' standard, i.e., a smear of $EB_4$ cells not exposed to/...

to $^{3}$H-TdR, was included as a control in all experiments.

Background grain counting for autoradiographs

This was estimated by scoring the frequency of the number of grains overlying each nucleus in a population of 500 live cells ( as assessed morphologically in smears) on duplicate slides i.e., 1,000 cells/test.

Measurement of LI, MI and PLM

For each time point (unless indicated otherwise in 'Results' section), the pulse- and continuous-LI was assessed by scoring 2,000 live cells as labelled or unlabelled; 500 cells were counted on duplicate slides for each test i.e.,1,000 cells/test, and each test was performed in duplicate.

For each time point, the MI was assessed by scoring the number of mitoses in a population of 5,000 live cells; 1,250 cells were counted on duplicate slides for each test i.e.,2,500 cells/test, and each test was performed in duplicate.

For each time point, the PLM was assessed by scoring 200 mitoses as labelled or unlabelled; 50 mitoses were counted on duplicate slides for each test i.e.,100 mitoses/test, and each test was performed in duplicate.

Slides were scored 'blind' for each experiment and several slides were recounted by an independent observer to check the reliability of results.

RESULTS/...

## RESULTS

### Effect of a 30 min. pulse of $^{3}$H-TdR on the growth rate of $EB_4$ cells

The effect of pulse-labelling on the growth rate of $EB_4$ cells using various concentrations of $^{3}$H-TdR is shown in fig. 45. This preliminary experiment was necessary so that the PLM experiment, which involves pulse-labelling with $^{3}$H-TdR, could be performed with a suitable concentration of $^{3}$H-TdR such that the growth rate was not affected over a period of 96hr. These results show a $^{3}$H-TdR dose-dependent effect on the growth rate; by 48hr after pulse-labelling cells treated with 5.0 $\mu$Ci/ml show a rapid and marked decline in growth rate, this being less marked with 2.0 and 1.0 $\mu$Ci/ml and non-existent with 0.5 and 0.1 $\mu$Ci/ml; by 96hr after pulse-labelling, all concentrations of $^{3}$H-TdR show some effect on the growth rate, though 0.5 and 0.1 $\mu$Ci/ml show a reduction in growth rate of $<10\%$ of the control value; by 144hr, all concentrations show a further decline in the growth rate. From these results, 0.1 $\mu$Ci/ml was chosen as the optimum concentration of $^{3}$H-TdR suitable for the PLM experiment (for further details on this preliminary experiment see Appendix II, p. 182).

### Effect of continuous-labelling with $^{3}$H-TdR on the growth rate and viability of both control and $10^{-5}$M MPS-treated $EB_4$ cells

The effect of continuous-labelling on the viability and growth rate of $EB_4$ cells using various concentrations of $^{3}$H-TdR is shown in figs. 46 and 47 respectively; both control and $10^{-5}$M MPS-treated $EB_4$ cells were tested after 96hr in culture, i.e.,once growth inhibition with the MPS was observed. After 10 days incubation with 0.1 and 0.05 $\mu$Ci/ml $^{3}$H-TdR a marked decrease in viability was noted/...

Fig. 45

Effect of $^{3}$H-TdR pulse-labelling on the growth rate of $EB_4$ cells

$EB_4$ cells were labelled for 30 min. at 37°C with various concentrations of $^{3}$H-TdR (0.1 - 5.0 µCi/ml) (treated) or pulsed with water (control), washed three times in ice-cold Hepes-buffered Hanks solution and resuspended in fresh growth medium for a further 144hr. $^{3}$H-TdR concentrations: ▽——▽, 0.1 µCi/ml; □——□, 0.5 µCi/ml; ◇——◇, 1.0 µCi/ml; ○——○, 2.0 µCi/ml; △——△, 5.0 µCi/ml.

Each point represents the mean of duplicate observations from one experiment.

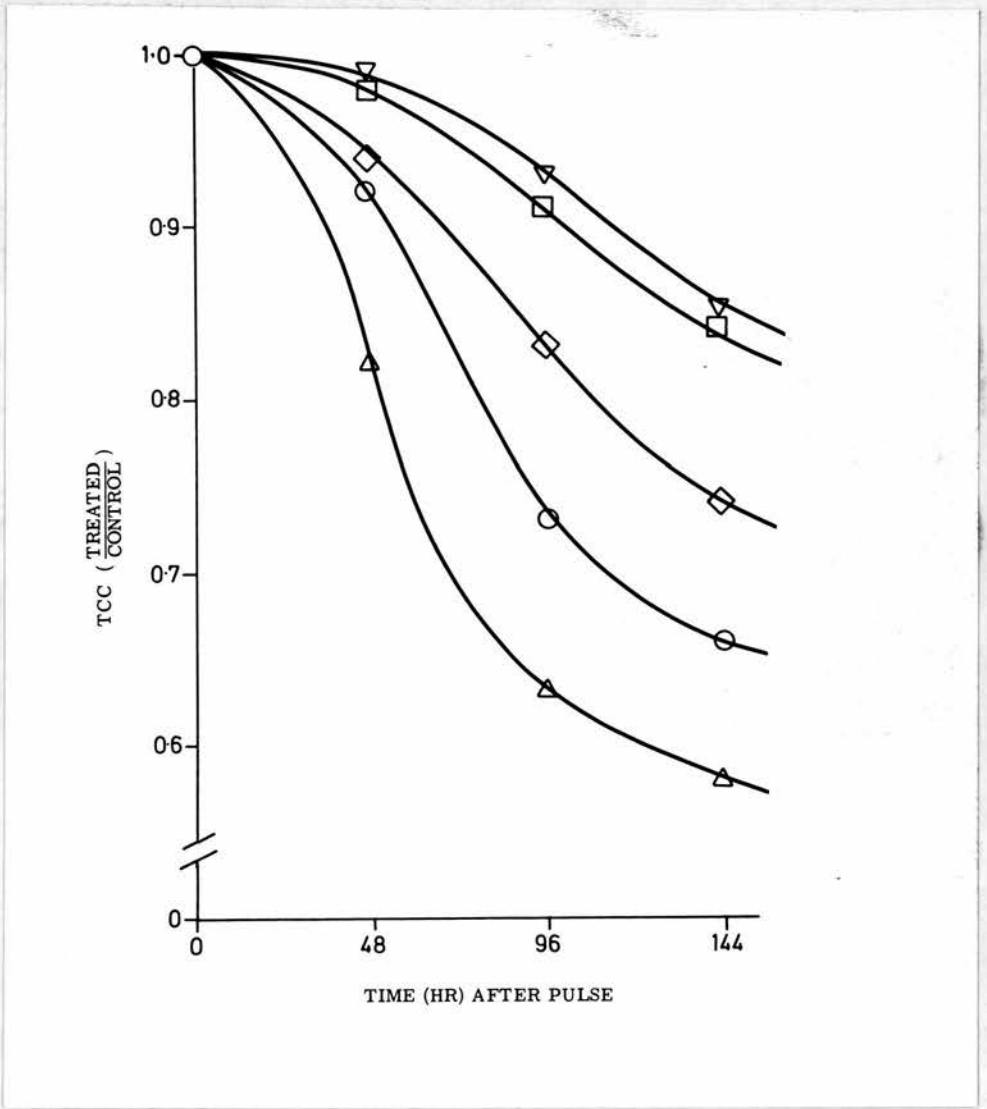


Fig. 46

Effect of $^{3}$H-TdR continuous-labelling on the viability of $10^{-5}$M MPS-treated and control $EB_4$ cells

After 96hr in culture (arrow), $10^{-5}$M MPS-treated and control (water) cultures of $EB_4$ cells were labelled continuously with various concentrations of $^{3}$H-TdR (a) 0.1 µCi/ml, (b) 0.05 µCi/ml, (c) 0.01 µCi/ml, (d) 0.005 µCi/ml. Cultures were assessed for viability and fed with fresh growth medium every 48hr. ●——●, control $EB_4$; ■——■, $10^{-5}$M MPS-treated $EB_4$; O- - - -O, control $EB_4$ + $^{3}$H-TdR; □- - - -□, $10^{-5}$M MPS-treated $EB_4$ + $^{3}$H-TdR. Each point represents the mean of duplicate observations from one experiment.

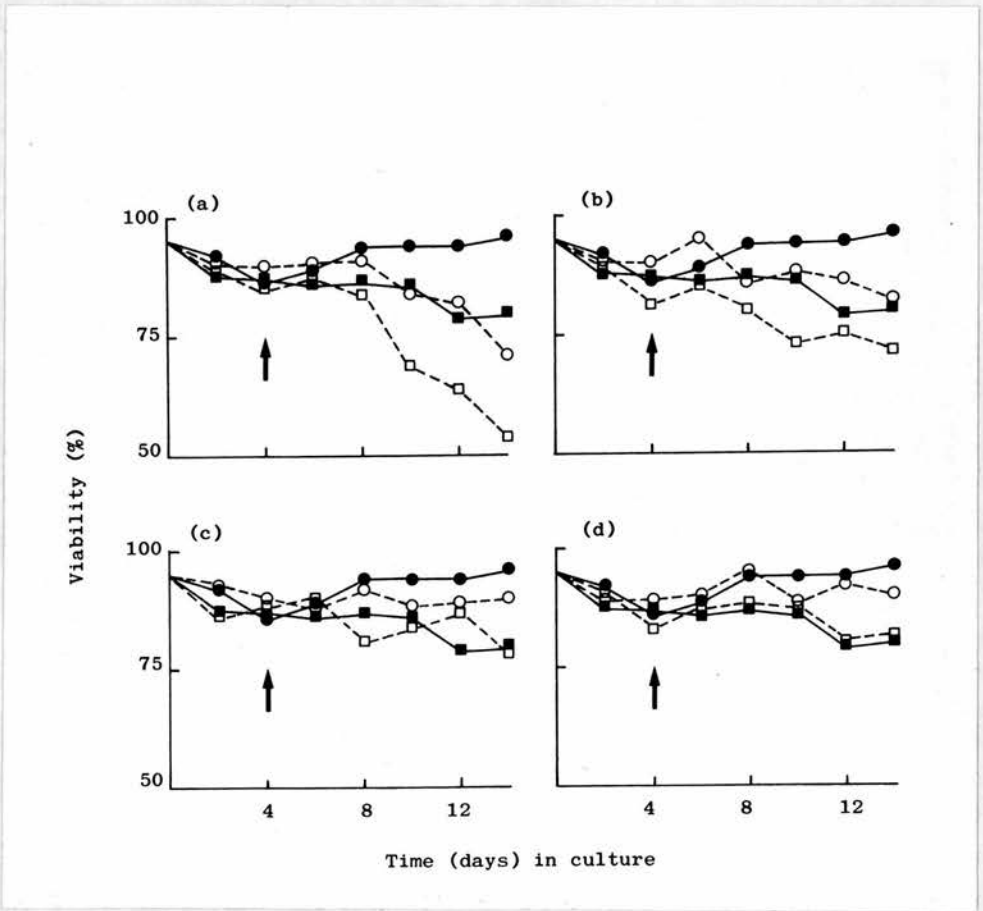
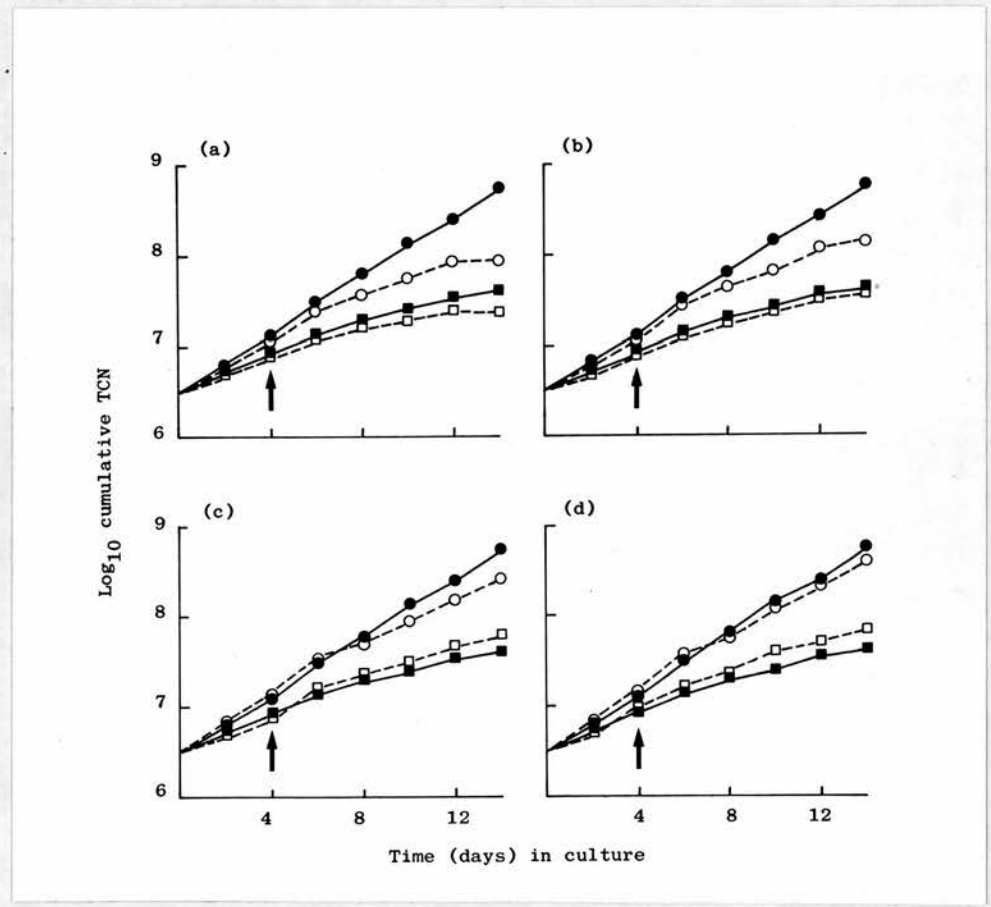


Fig. 47

Effect of $^{3}$H-TdR continuous-labelling on the growth rate of $10^{-5}$M MPS-treated and control $EB_4$ cells

After 96hr in culture (arrow), $10^{-5}$M MPS-treated and control (water) cultures of $EB_4$ cells were labelled continuously with various concentrations of $^{3}$H-TdR (a) 0.1 μCi/ml, (b) 0.05 μCi/ml, (c) 0.01 μCi/ml, (d) 0.005 μCi/ml. Cultures were assessed for total cell concentration and fed with fresh growth medium every 48hr. ●——●, control $EB_4$; ■——■, $10^{-5}$M MPS-treated $EB_4$; O- - - -O, control $EB_4$ + $^{3}$H-TdR; □- - - -□, $10^{-5}$M MPS-treated $EB_4$ + $^{3}$H-TdR. Each point represents the mean of duplicate observations from one experiment.



noted with both treated and control $EB_4$ cells - this was not evident with 0.01 and 0.005 $\mu$Ci/ml. With control $EB_4$ cells, a drop in growth rate (as illustrated by a plot of log cummulative total cell number) was observed with 0.1, 0.05 and 0.01 $\mu$Ci/ml $^3$H-TdR, this being evident after 4 days incubation with 0.1 and 0.05 $\mu$Ci/ml and after 6 days with 0.01 $\mu$Ci/ml; there was no observed decrease in growth rate with 0.005 $\mu$Ci/ml. With $10^{-5}$M MPS-treated cells, only 0.1 $\mu$Ci/ml showed any marked decrease in growth rate.

This preliminary experiment was undertaken so that a continuous-LI experiment could be performed on $EB_4$ cells with a concentration of $^3$H-TdR which showed no deleterious effects on either the growth rate or the viability of the cells. The concentration of 0.005 $\mu$Ci/ml would seem ideal - however such a low concentration would require an extremely long exposure time for detection of labelled cells on autoradiographs. As this was not feasible, 0.01 $\mu$Ci/ml was therefore used in further experiments as this concentration showed no decrease in viability and the growth rate was not affected until 6 days after incubation with the $^3$H-TdR.

Effect of $10^{-5}$M MPS on the $^3$H-TdR pulse-LI of $EB_4$ cells

Typical autoradiographs of $EB_4$ cells pulse-labelled for 30 min. with 1.0 $\mu$Ci/ml $^3$H-TdR are shown in fig. 48(a); fig. 48(b) illustrates a 'cold' standard. The frequency distribution of nuclear grain counts (fig. 49) revealed that all cells with $> 5$ grains overlying the nucleus could be scored as labelled. The majority of the population showed $> 50$ grains/nucleus.

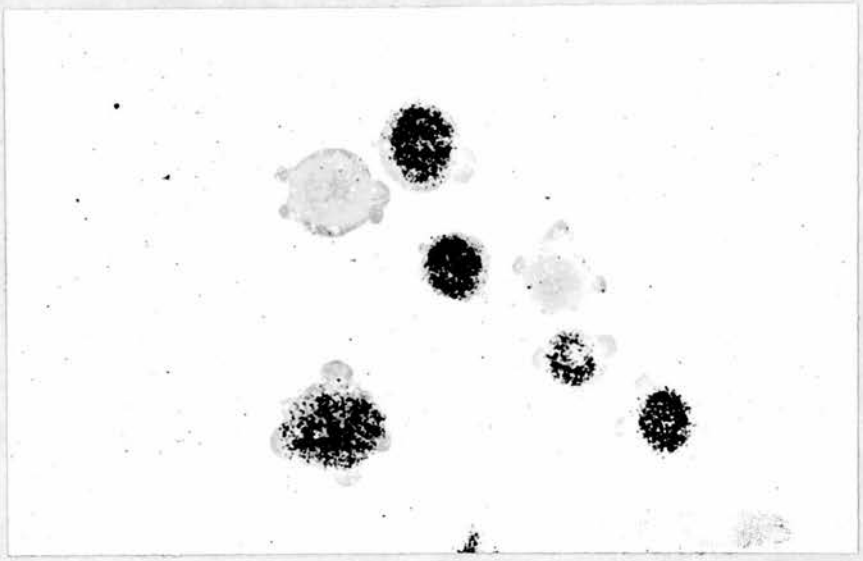
Results/...

Fig. 48

Autoradiographs of control $EB_4$ cells

(a) $EB_4$ cells pulse-labelled with 1.0 µCi/ml $^3$H-thymidine ($^3$H-TdR) for 30 min. at 37°C. Cells containing radioactively labelled DNA in their nucleus (i.e., S-phase cells) have black grains overlying them. (b) a 'cold' standard of $EB_4$ cells not exposed to $^3$H-TdR. G. x 510.

(a)



(b)

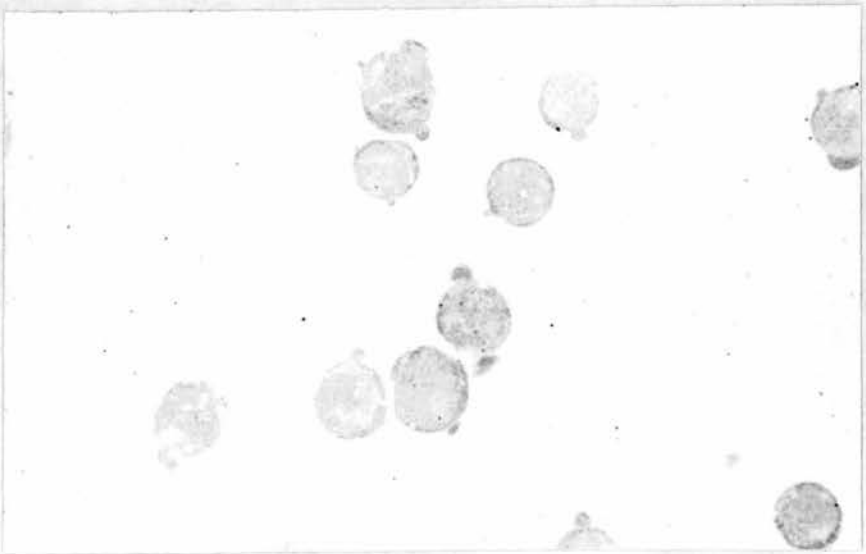
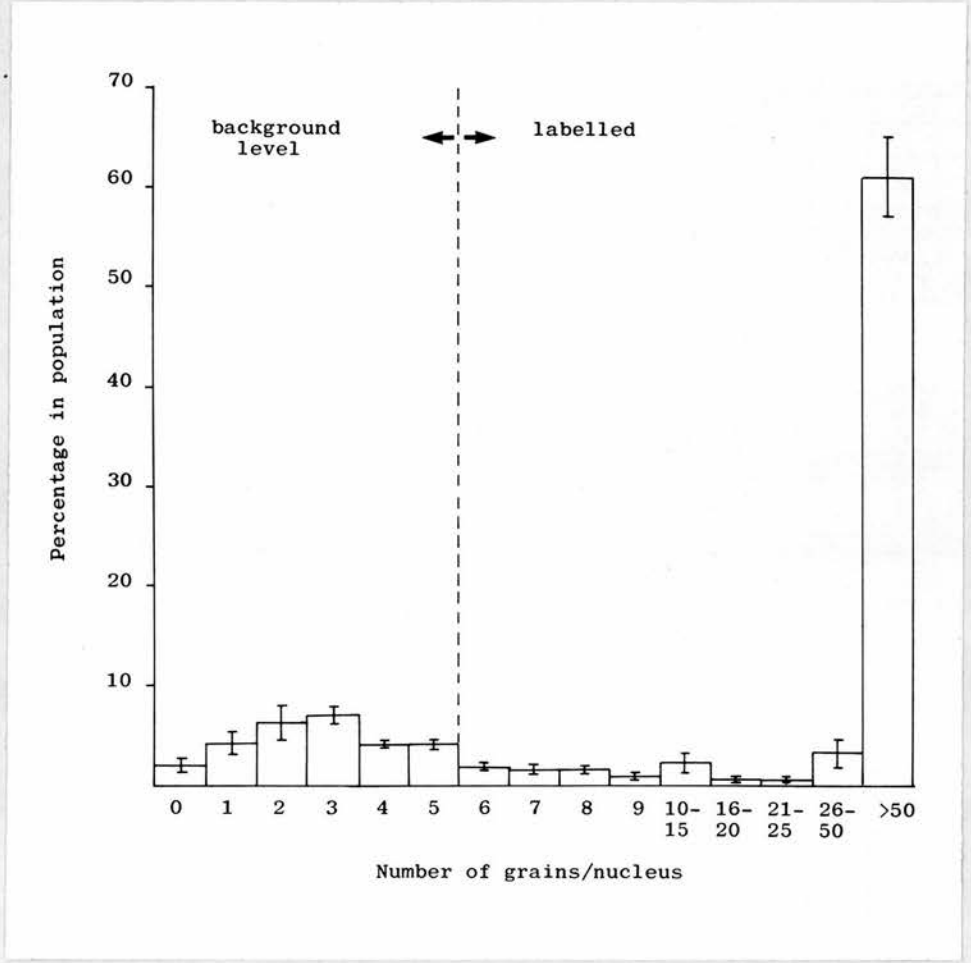


Fig. 49

Frequency distribution histogram of nuclear grain counts from autoradiographs of control $EB_4$ cells pulse-labelled with 1.0 µCi/ml $^3$H-thymidine for 30 min. at 37°C

After pulse-labelling, smears of cells were prepared for autoradiography. 500 cells were counted/autoradiograph and the number of grains over each nucleus grouped together as shown. Grain count distribution is clearly bimodal and the background level was chosen to include the majority of cells in the lightly labelled sub-population, with a mode of 2 - 3 grains (as indicated).

Each bar represents the mean ± range of counts from 2 typical autoradiographs.



Results of the effect of $10^{-5}$M MPS on the pulse-LI of $EB_4$ cells are shown in fig. 50. The pulse-LI of control $EB_4$ cells remained at 62% for the duration of the experiment. With $10^{-5}$M MPS-treated cells, the pulse-LI fell immediately after treatment to reach a new stable level of 39% i.e., a fall of 23%. The mean length of time for the level to decrease to 39% can be calculated from the inflection point between the falling arm of the curve and the new plateau level where the mean = 33hr (range 23hr - 58hr) (see fig. 50).

Effect of $10^{-5}$M MPS on the mitotic index (MI) of $EB_4$ cells

Results are shown in fig. 51. The difference in MI values between control and $10^{-5}$M MPS-treated cells increases from about 12hr after addition of MPS and this reaches a new plateau level after about 42hr where the treated cells show a MI approximately 0.8% lower than that of the control cells.

Effect of $10^{-5}$M MPS on the duration of the cell cycle time and the individual phases of the cell cycle of $EB_4$ cells - PLM technique using $^3$H-TdR pulse-label

The well-known conventional technique of PLM (Quastler and Sherman, 1959; Baserga and Wiebel, 1969; Mitchison, 1971; Aherne, Camplejohn and Wright, 1977; Steel, 1977) was used to determine the duration of the cell cycle and its component phases (see Appendix III(a), p. 184 for theoretical details) - this involves drawing a curve of best fit by eye through the individual data points. In addition, data was also fed through a Monte Carlo computer programme devised by Barrett (Barrett, 1966; Steel and Hanes, 1971) this being kindly carried out by/...

Fig. 50

Effect of $10^{-5}$M methylprednisolone (MPS) on the $^{3}$H-thymidine ($^{3}$H-TdR) pulse-labelling index (LI) of $EB_4$ cells

At the time intervals shown during treatment, samples of control (water) and $10^{-5}$M MPS-treated $EB_4$ cells were pulse-labelled with 1.0 µCi/ml $^{3}$H-TdR for 30 min. at 37°C - smears of cells were then prepared for autoradiography. Cell nuclei with > 5 grains were scored as labelled. The number of labelled cells for each point is expressed as a percentage of a total of 2,000 cells counted. ●——●, control $EB_4$ cells; O——O, $10^{-5}$M MPS-treated $EB_4$ cells. - - - -, indicates the point of inflection of the 2 arms of the curve.

Each point represents the mean ± 1S.E. of 2 experiments each with duplicate observations.

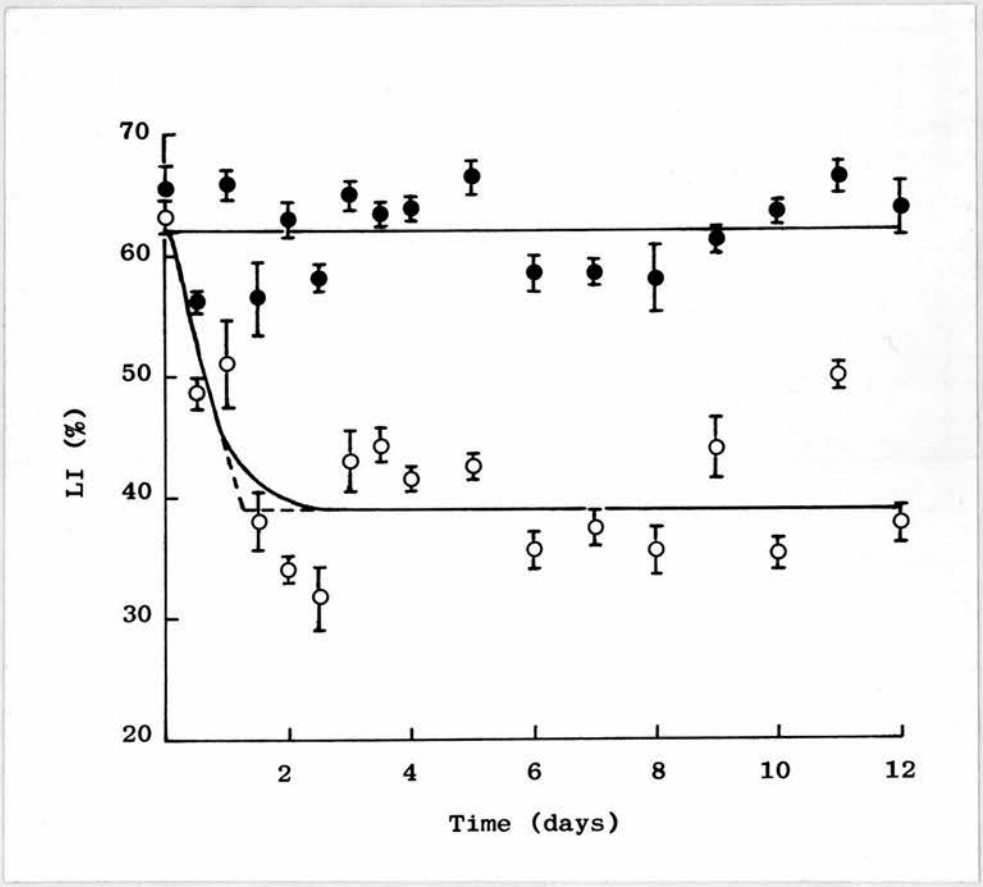
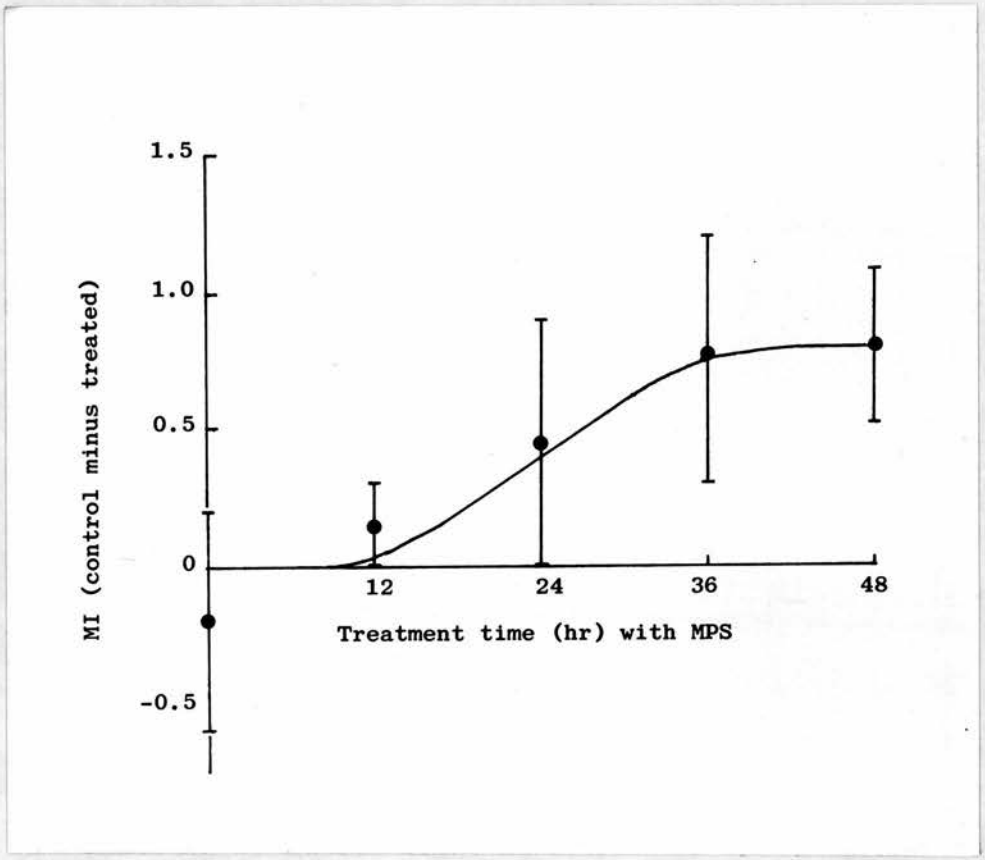


Fig. 51

Effect of $10^{-5}$M methylprednisolone (MPS) on the mitotic index (MI) of $EB_4$ cells

At the time intervals shown during treatment, smears of both control (water) and MPS-treated $EB_4$ cells were prepared and stained with Giemsa. The number of mitotic cells for both control and treated cells is expressed as a percentage of a total of 5,000 cells counted.

Each point represents the mean (control minus treated) ± range of 2 experiments each with duplicate observations.



by Dr. G.G. Steel; this fits a PLM curve from which cell cycle parameters are derived, and also computes the distribution of cell cycle times. These parameters are subsequently denoted in the text as follows:

$t_C$ = cell cycle time

$t_{G1}$ = duration of $G_1$ phase

$t_S$ = duration of S phase

$t_{G2}$ = duration of $G_2$ phase

$t_M$ = duration of mitotic phase

The PLM data was assessed on autoradiographs in serial samples taken over a period of 96hr. Typical examples of such labelled or unlabelled mitoses are shown in fig. 52. The frequency distribution of nuclear grain counts (fig. 53) revealed that all mitotic cells with $>4$ grains overlying the chromosomes could be scored as labelled in both $10^{-5}$M MPS-treated and control $EB_4$ cells. The majority of the labelled cells showed $>25$ grains/nucleus.

The PLM curves for control and $10^{-5}$M MPS-treated $EB_4$ cells as drawn by eye are shown in figs. 54 and 55 respectively. With control curves, at least 4 waves of labelled mitoses are seen, but with treated curves there is damping after the third wave to a level of about 56% which suggests more variation from the control cells in $t_C$ and/or its phases.

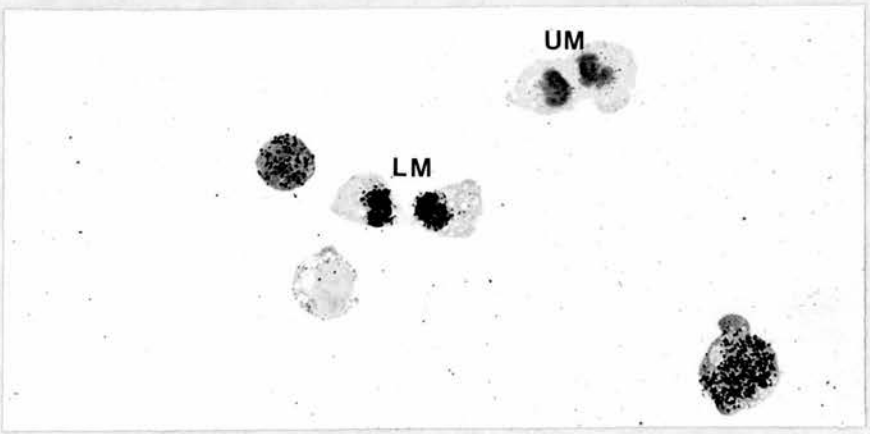
The duration of $t_C$ and its component phases as derived from these curves (see Appendix III(a), p. 184) are presented in table 9 (see p. 132) and individual parameters are found to be similar for both/...

Fig. 52

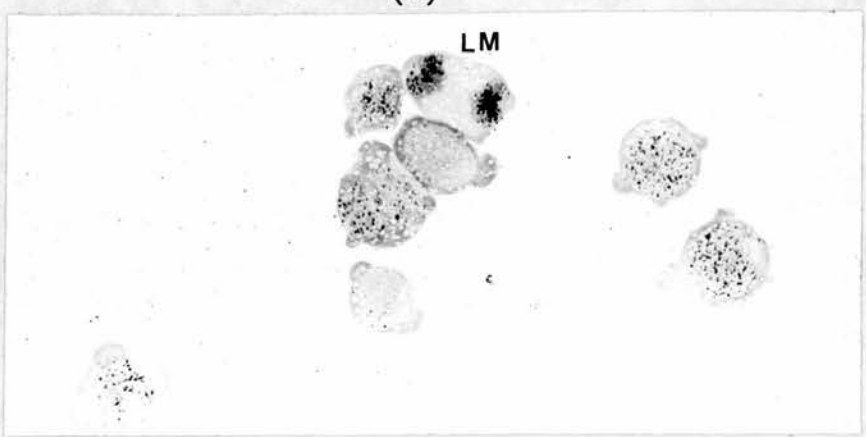
Autoradiographs of control $EB_4$ cells

Cells were pulse-labelled for 30 min. at 37°C with 0.1 µCi/ml $^3$H-thymidine ($^3$H-TdR), washed three times and resuspended in fresh growth medium containing no $^3$H-TdR. Cultures were sampled and smears of cells prepared for autoradiography at (a) 8hr and (b), (c) 36hr after pulsing. LM, labelled mitosis; UM, unlabelled mitosis. G. x 510.

(a)




(b)




(c)

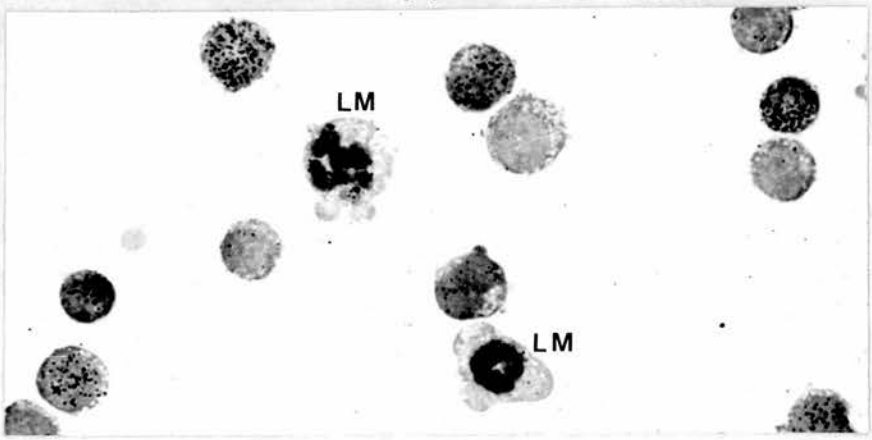


Fig. 53

Frequency distribution histograms of nuclear grain counts from autoradiographs of control and $10^{-5}$M methylprednisolone (MPS)-treated $EB_4$ cells pulse-labelled with 0.1 µCi/ml $^3$H-thymidine ($^3$H-TdR)

Control (water) and $10^{-5}$M MPS-treated $EB_4$ cells were pulse-labelled for 30 min. at 37°C, washed three times and resuspended in fresh growth medium containing no $^3$H-TdR; treatment with water and $10^{-5}$M MPS was maintained. Cultures were sampled at 8hr after pulsing and smears of cells prepared for autoradiography: (a) control $EB_4$ cells, (b) $10^{-5}$M MPS-treated $EB_4$ cells. 500 cells were counted/autoradiograph and the number of grains over each nucleus grouped together as shown. Grain count distribution is clearly bimodal and the background level was chosen to include the majority of cells in the lightly labelled sub-population (as indicated).

Each bar represents the mean ± range of counts from 2 typical autoradiographs for both control and $10^{-5}$M MPS-treated cells.

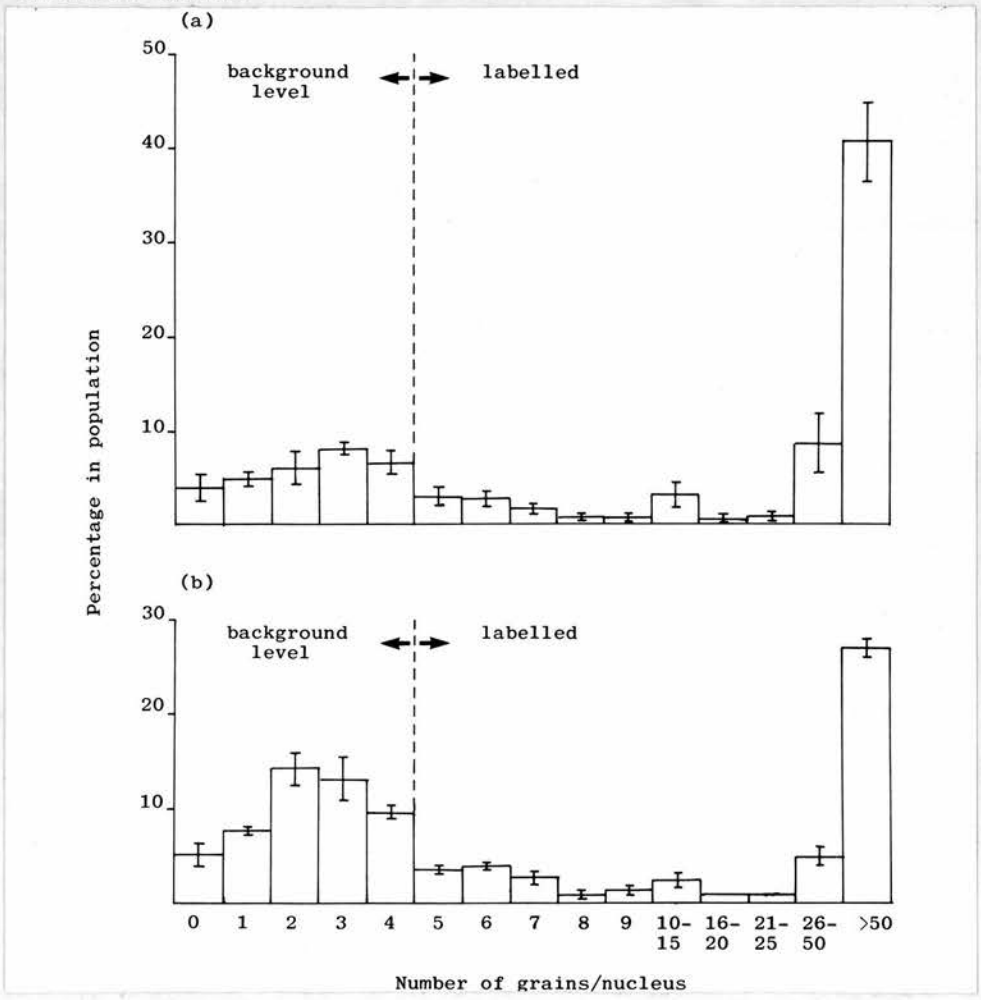


Fig. 54

Percentage of labelled mitoses (PLM) curve for control $EB_4$ cells

After 6 days in culture, control (water) $EB_4$ cells were pulse-labelled for 30 min. at 37°C with 0.1 µCi/ml $^3$H-thymidine ($^3$H-TdR), washed three times and resuspended in fresh growth medium containing no $^3$H-TdR; treatment with water was maintained. Samples were removed at the time intervals shown after pulsing and smears prepared for autoradiography. Mitotic cells with $>4$ grains overlying the chromosomes were scored as labelled. The number of labelled mitoses for each point is expressed as a percentage of a total of 200 mitoses counted.

Each point represents the mean $\pm$ 1S.E. of 4 separate observations from one experiment.

The curve is drawn by eye as a best fit to the data points. The durations of the cell cycle ($t_C$) and its phases ($t_{G1}$, $t_S$, $t_{G2}$ and $t_M$) are calculated from this curve as described in Appendix III(a), p. 184 or in the text p. 132.

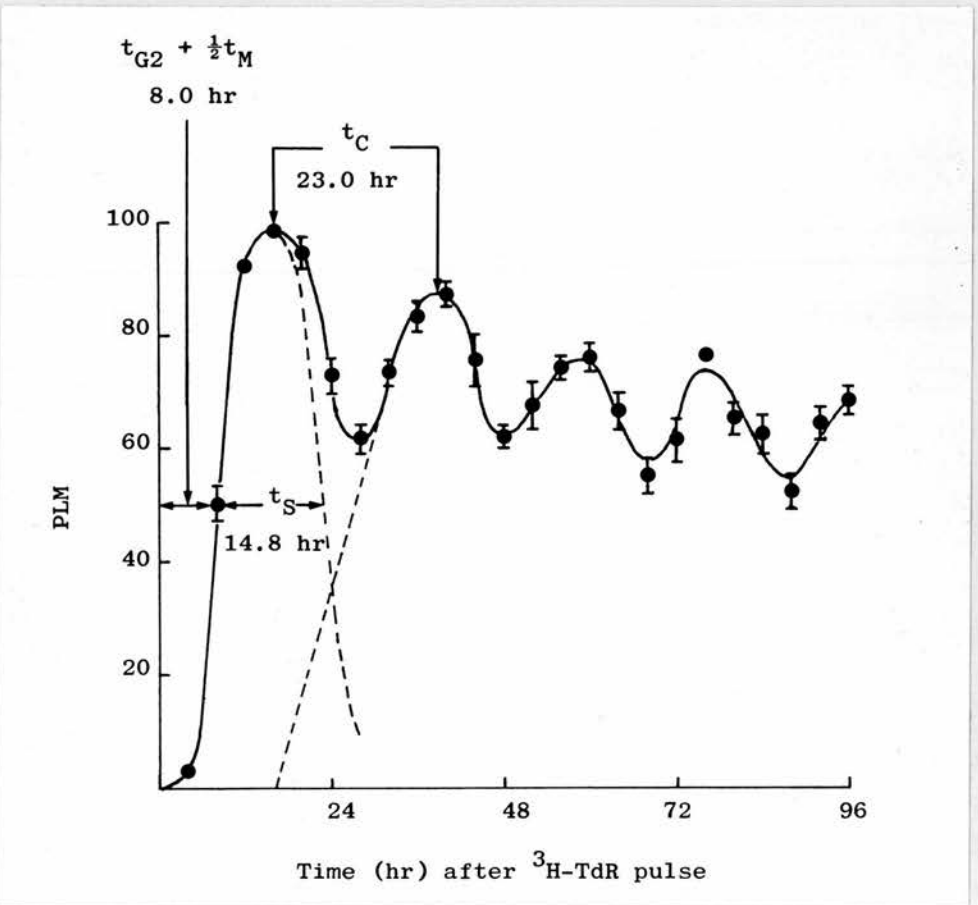


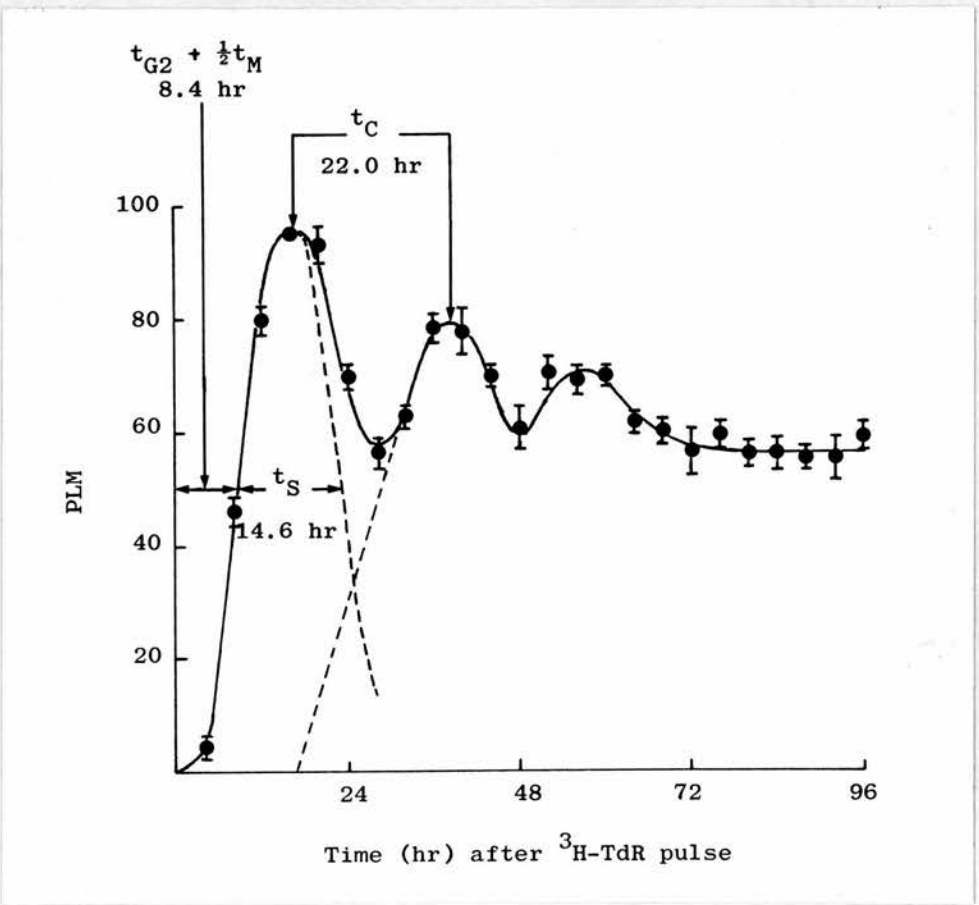
Fig. 55

Percentage of labelled mitoses (PLM) curve for $10^{-5}$M methylprednisolone (MPS)-treated $EB_4$ cells

After treatment with $10^{-5}$M MPS for 6 days, $EB_4$ cells were pulse-labelled for 30 min. at 37°C with 0.1 μCi/ml $^3$H-thymidine ($^3$H-TdR), washed three times and resuspended in fresh growth medium containing $10^{-5}$M MPS, but no $^3$H-TdR. Samples were removed at the time intervals shown after pulsing and smears prepared for autoradiography. Mitotic cells with $>4$ grains overlying the chromosomes were scored as labelled. The number of labelled mitoses for each point is expressed as a percentage of a total of 200 mitoses counted.

Each point represents the mean $\pm$ 1S.E. of 4 separate observations from one experiment.

The curve is drawn by eye as a best fit to the data points. The duration of the cell cycle ($t_C$) and its phases ($t_{G1}$, $t_S$, $t_{G2}$ and $t_M$) are calculated from this curve as described in Appendix III(a), p. 184 or in the text p. 132.



both control and treated cells. Unfortunately the first trough in the PLM curves does not fall to below the 50% level - this could be due to the selected background grain count ($>4$ grains) being too low, or again a very marked variation in $t_C$ and its phases. As a consequence, $t_S$ can only be roughly estimated by visual methods: a notional descending portion of the first wave was constructed as shown in figs. 54 and 55 by extrapolating the leading edge of the second wave back to the mid-point of the first wave and then subtracting this from the trailing edge of the first wave. For measurement of $t_M$ ($t_M = \frac{MI}{LI} \times t_S$ - see Appendix III(a), p. 185) the MI and LI were measured from the 4hr sampling point: MI = 1.84% and 0.97% for control and $10^{-5}$M MPS-treated cells respectively; LI = 59.7% and 35.1% for control and $10^{-5}$M MPS-treated cells respectively.

TABLE 9 ESTIMATION OF THE DURATION OF THE CELL CYCLE AND ITS COMPONENT PHASES FOR CONTROL (WATER) AND $10^{-5}$M METHYLPREDNISOLONE (MPS)-TREATED $EB_4$ CELLS FROM THE PERCENTAGE OF LABELLED MITOSES CURVES (FIGS. 54 AND 55) (USING $^3$H-TdR PULSE-LABELLING TECHNIQUE)

| Cell cycle parameter | Duration (hr) for: | |
|---|---|---|
| | Control $EB_4$ cells | $10^{-5}$M MPS-treated $EB_4$ cells |
| $t_C$ | 23.0 | 22.0 |
| $t_{G2}$ | 7.8 | 8.2 |
| $t_S$ | 14.8 | 14.6 |
| $t_{G1}$ | -0.1* | -1.2* |
| $t_M$ | 0.5 | 0.4 |

*the negative values for $t_{G1}$ are accounted for in the sub-section on

continuous/...

continuous-labelling with $^{3}$H-TdR - see table 11, p. 140.

The computed PLM curves are shown in figs. 56 and 57 for control and $10^{-5}$M MPS-treated cells respectively. There appears to be a marked discrepancy from the hand-drawn curves, as in both control and $10^{-5}$M MPS-treated cells the computer does not simulate the second and subsequent peaks of the curve - this arises, however, as the programme incorporates sufficient spread in $G_2$ and sufficiently long values of $t_{G2}$ and $t_S$ from the first peak data to render it impossible to fit a curve that can rise again to simulate the early second peak of the data (G.G. Steel, personal communication). This possibly indicates that the first peak may be prolonged or delayed due to a disturbance by the presence of $^{3}$H-TdR or perhaps to the technique of removing $^{3}$H-TdR from the cultures (vide infra). Figs. 56 and 57 also show the computed distribution of $t_C$ for both control and $10^{-5}$M MPS-treated cells respectively.

The duration of the cell cycle parameters as obtained from the computer analysis are shown in table 10, p. 136 (cf. table 9, p. 132), and again the duration of individual parameters are similar for control and $10^{-5}$M MPS-treated cells. However, as the curves do not fit the data points, then these measurements are not considered to be of any further value.

TABLE 10/...

Fig. 56

Monte Carlo computer analysis of percentage of labelled mitoses (PLM) data for control $EB_4$ cells

(a) PLM curve. - - - -, computed; ——, drawn by eye through data points as previously shown in fig. 54 (this is included for comparison). (b) computed distribution of cell cycle times. (See legend to fig. 54 for experimental details.)

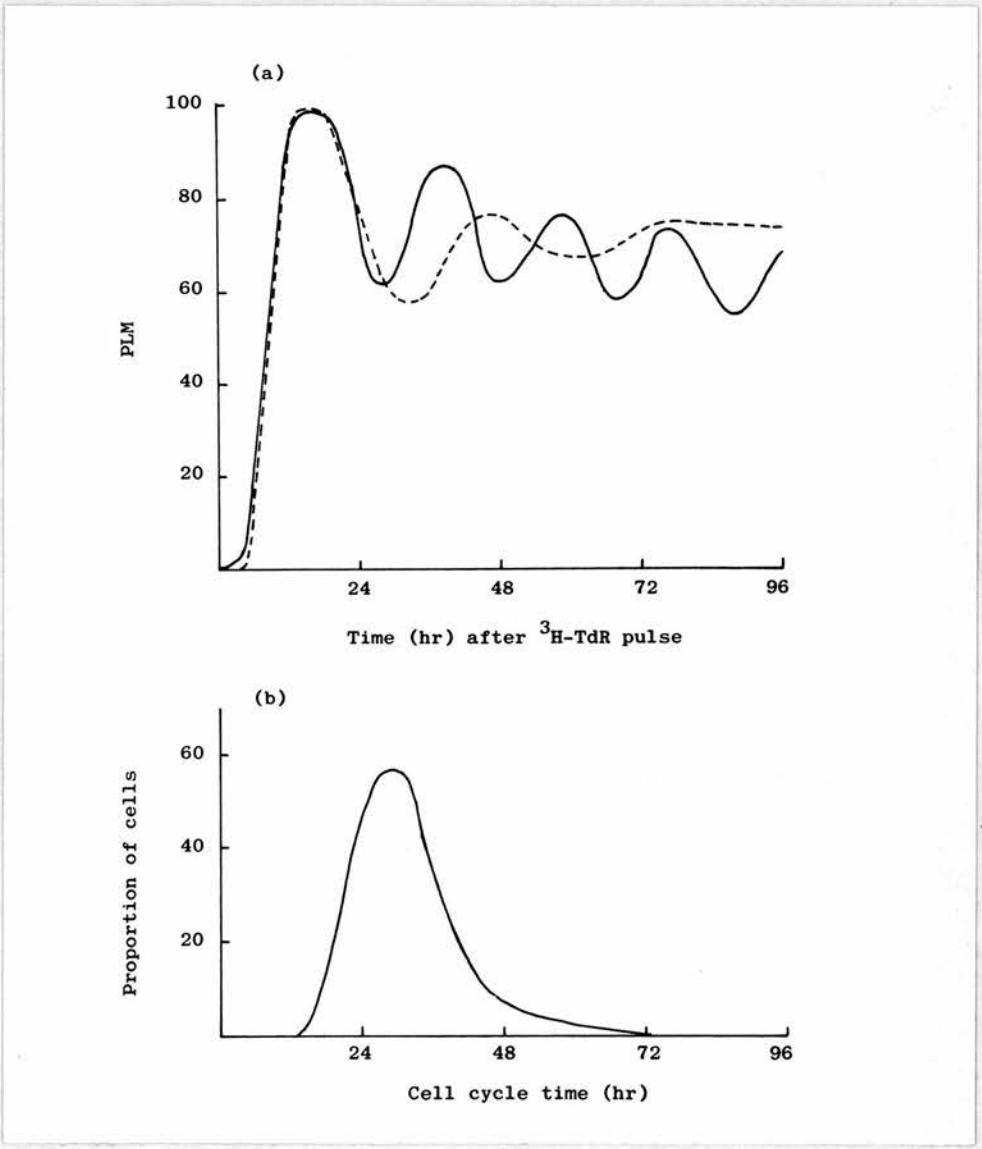


Fig. 57

Monte Carlo computer analysis of percentage of labelled mitoses (PLM) data for $10^{-5}$M methylprednisolone-treated $EB_4$ cells

(a) PLM curve. - - - -, computed; ——— , drawn by eye through data points as previously shown in fig. 55 (this is included for comparison). (b) computed distribution of cell cycle times. (See legend to fig. 55 for experimental details.)

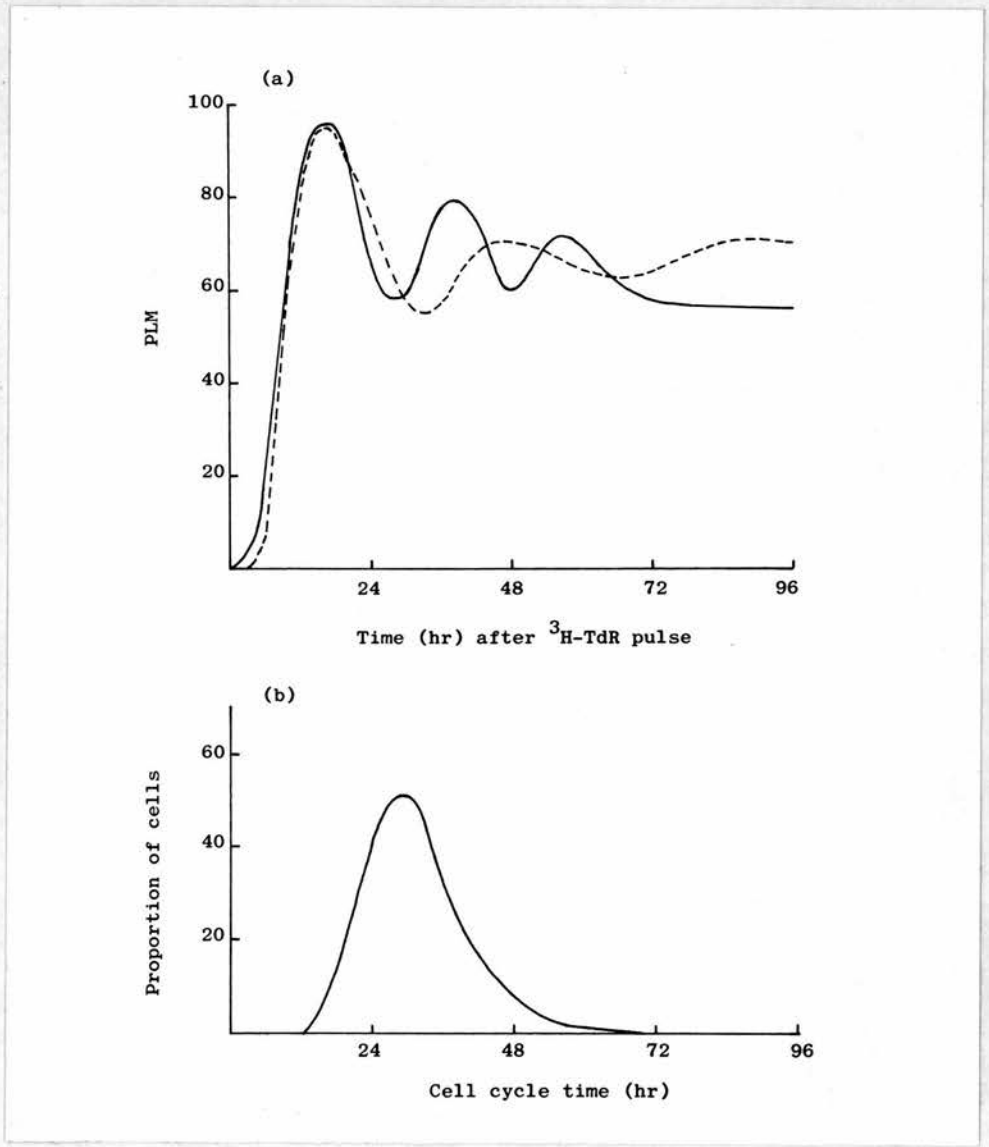


TABLE 10 ESTIMATION OF THE DURATION OF THE CELL CYCLE AND ITS COMPONENT PHASES FOR CONTROL (WATER) AND $10^{-5}$M METHYLPREDNISOLONE (MPS)-TREATED $EB_4$ CELLS AS OBTAINED FROM THE COMPUTED PERCENTAGE OF LABELLED MITOSES DATA (FIGS. 56 AND 57)

| Cell cycle parameter | Median duration (hr) for: | |
|---|---|---|
| | Control $EB_4$ cells | $10^{-5}$M MPS-treated $EB_4$ cells |
| $t_C$ | 29.7 | 29.5 |
| $t_{G2}$ | 8.2 | 8.9 |
| $t_S$ | 20.8 | 19.8 |
| $t_{G1}$ | 0.5 | 0.3 |
| $t_M$ | 1.2 | 0.5 |

Effect of $10^{-5}$M MPS on the growth fraction ($f_G$) of $EB_4$ cells as measured from the PLM pulse-labelling curves

There are 2 well-recognised methods of measuring $f_G$ from the PLM pulse-labelling curves:

(a) $f_G = \frac{LI}{<PLM>}$ (Mendelsohn, 1962)

(b) $\ln (1 + f_G) = \frac{t_C}{t_{C(pot)}} \times \ln 2$ (Steel, 1968)

(see Appendix III(c), p. 190 for further details).

Unfortunately, as the PLM data could not be fitted accurately by computer model analysis (figs. 56 and 57) and as Steel's equations for calculating $f_G$ and $t_{C(pot)}$ (see Appendix III(c), p. 190) assume a conventional cell cycle model, then it was not considered worthwhile making/...

making a detailed computer analysis of these parameters (G.G. Steel, personal communication).

Thus, Mendelsohn's method alone was used to calculate $f_G$. $\langle PLM \rangle$ was estimated from averaging the values of PLM obtained every hour over one cell cycle duration, between the first and second peaks on the PLM curves in figs. 54 and 55:

$\langle PLM \rangle$, control cells = 79.1%

$\langle PLM \rangle$, $10^{-5}$M MPS-treated cells = 73.2%

Thus, $f_G$ values for control and $10^{-5}$M MPS-treated cells are found to be 75% and 48% respectively i.e., treated cells show a 27% decrease in $f_G$ value compared to the control cells.

Effect of $10^{-5}$M MPS on $t_{G2}$, $t_{G1}$ and $f_G$ as measured by $^3$H-TdR continuous-labelling techniques

Continuous-labelling with $^3$H-TdR is another conventional technique which may be used to determine $t_{G2}$, $t_{G1}$ and $f_G$ values by analysing the PLM and continuous-LI curves (see Appendix III(b), p. 185 for theoretical details.

For continuous-labelling experiments, the frequency distribution of nuclear grain counts on autoradiographs revealed that cells with $> 4$ grains overlying the nucleus could be scored as labelled in both control and $10^{-5}$M MPS-treated cultures (fig. 58).

The PLM curves for both control and $10^{-5}$M MPS-treated cultures (fig. 59) rose to 100% over a maximum period of about 16hr. The 50% level/...

Fig. 58

Frequency distribution histograms of nuclear grain counts from autoradiographs of $EB_4$ cells continuously-labelled for 1hr with 0.01 μCi/ml $^3H$-thymidine (a) control (water) $EB_4$ cells and (b) $10^{-5}$M methylprednisolone (MPS)-treated $EB_4$ cells

After labelling, smears of cells were prepared for autoradiography. 500 cells were counted/autoradiograph and the number of grains over each nucleus grouped together as shown. Grain count distribution is clearly bimodal and the background level was chosen to include the majority of cells in the lightly labelled sub-population (as indicated).
Each bar represents the mean ± range of counts from 2 typical autoradiographs for both control and $10^{-5}$M MPS-treated cells.

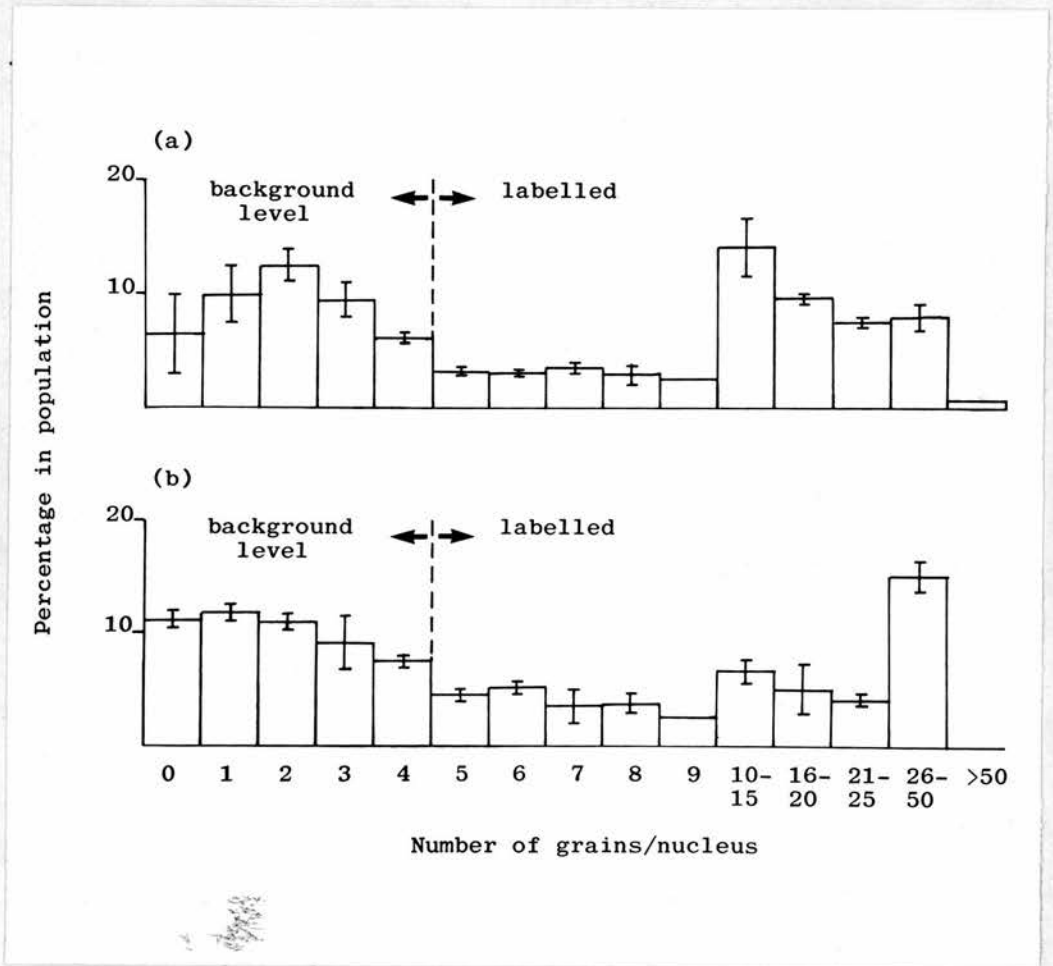
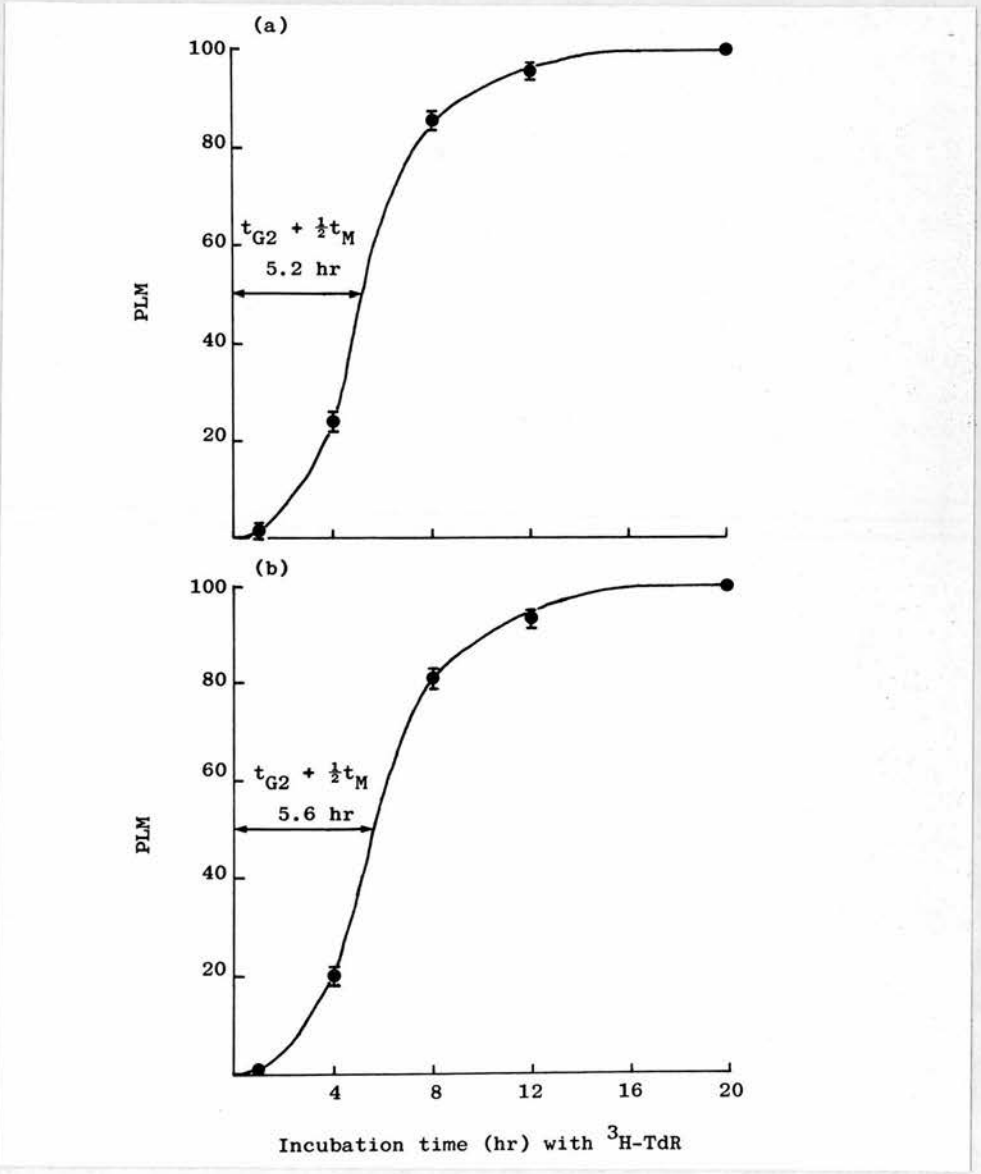


Fig. 59

Percentage of labelled mitoses (PLM) as a function of time for (a) control and (b) $10^{-5}$M methylprednisolone (MPS)-treated $EB_4$ cells, exposed continuously to 0.01 µCi/ml $^3$H-thymidine ($^3$H-TdR)

After treatment for 6 days, control (water) and $10^{-5}$M MPS-treated $EB_4$ cells were continuously labelled with 0.01 µCi/ml $^3$H-TdR. At the time intervals shown after addition of $^3$H-TdR, samples were removed and smears of cells prepared for autoradiography. Mitotic cells with $>4$ grains overlying the chromosomes were scored as labelled. The number of labelled mitoses for each point is expressed as a percentage of a total of 200 mitoses counted.

Each point represents the mean $\pm$ 1S.E. of 2 experiments each with duplicate observations. The duration of $t_{G2} + \frac{1}{2}t_M$ is calculated as shown.



level showed a mean $t_{G2} + \frac{1}{2}t_M$ value of 5.2hr and 5.6hr for control and treated cultures respectively. These $t_{G2}$ values are lower than those found by the pulse-labelling PLM technique (see table 9, p. 132 ); this possibly arises as the pulse-labelling PLM method involves excessive disturbance of the cells due to the washing and centrifuging procedure required to remove the excess $^3$H-TdR. It is thought that this might cause a transient delay or blockage of cells in $G_2$ or at the $G_2$/S interphase such that $t_{G2}$ is overestimated (see 'Comments', p. 147). Thus the lower $t_{G2}$ values obtained from the continuous-labelling PLM curve (where no disturbance of cells occurs) have been used together with the values for $t_C$ and $t_S$ already obtained from the pulse-labelling PLM data to devise the cell cycle phase data shown in table 11.

TABLE 11 ESTIMATION OF THE DURATION OF THE CELL CYCLE AND ITS COMPONENT PHASES FOR CONTROL (WATER) AND $10^{-5}$M METHYLPREDNISOLONE (MPS)-TREATED $EB_4$ CELLS FROM THE PERCENTAGE OF LABELLED MITOSES CURVES USING BOTH $^3$H-TdR PULSE-LABELLING AND CONTINUOUS-LABELLING TECHNIQUES

| Cell cycle parameter | Duration (hr) for: | |
|---|---|---|
| | Control $EB_4$ cells | $10^{-5}$M MPS-treated $EB_4$ cells |
| $t_C$ | 23.0 | 22.0 |
| $t_{G2}$ | 4.9* | 5.4* |
| $t_S$ | 14.8 | 14.6 |
| $t_{G1}$ | 2.8* | 1.6* |
| $t_M$ | 0.5 | 0.4 |

*$t_{G2}$, and by substitution $t_{G1}$ values obtained from continuous-labelling PLM curve - compare table 9, p. 132 .

The/...

The continuous-LI curves obtained for control and $10^{-5}$M MPS-treated $EB_4$ cells are shown in fig. 60. As expected both curves show an initial lag phase over a period of time equivalent to $t_{G2}$ (as measured from PLM continuous-labelling curves - fig. 59). It appears from the upward slopes of the curves that entry of cells into S is slightly faster for control than $10^{-5}$M MPS-treated cells - however such a result is highly subjective as curves are only drawn by eye through data points.

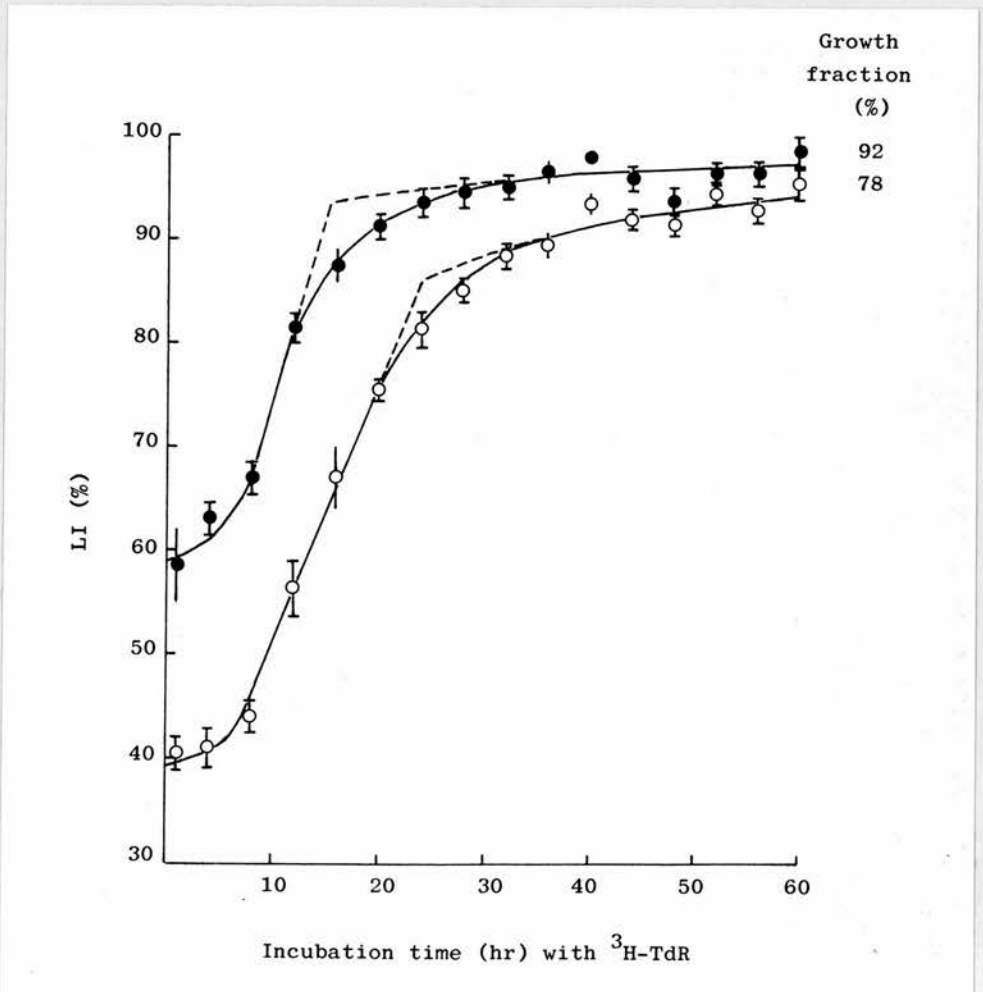
The LI values for control and $10^{-5}$M MPS-treated cultures increase from 59% and 39% respectively, values similar to those obtained from $^3$H-TdR pulse-LI experiments (cf. fig. 50); both curves asymptotically approach 100% labelling. From these curves $f_G$ values can be calculated by extrapolating the 'slowly rising' portion back to the end of $G_2$ by eye (see Appendix III(b), p. 185) - this method, however, only gives a rough estimate as it depends on which part of the 'slowly rising' curve is chosen for extrapolation. Accepting such limitations, $f_G$ values for control and $10^{-5}$M MPS-treated cells are calculated as 92% and 78% respectively (see fig. 60), i.e., treated cells show a 14% decrease in $f_G$. Qualitatively this is in agreement with PLM pulse-labelling data where the $f_G$ value for treated cells was lower than that of control cells: however a quantitative discrepancy might be expected considering the inherent errors present in both techniques for calculating $f_G$. A rough estimate of the mean $t_{G1} + t_M + t_{G2}$ values can also be calculated from these curves (see fig. 60): the mean $t_{G1} + t_M + t_{G2}$ value for control cells = 15.5hr (range 12 - 32hr) and for $10^{-5}$M MPS-treated cells = 23.5hr (range 20 - 36hr) i.e., the treated cells show an <u>apparent</u> increase in/...

Fig. 60

Effect of $10^{-5}$M methylprednisolone (MPS) on the $^{3}$H-thymidine ($^{3}$H-TdR) continuous-labelling index (LI) of $EB_4$ cells

After treatment for 6 days, control (water) and $10^{-5}$M MPS-treated $EB_4$ cells were continuously labelled with 0.01 µCi/ml $^{3}$H-TdR. At the time intervals shows after addition of $^{3}$H-TdR samples were removed and smears of cells prepared for autoradiography. Cell nuclei with > 4 grains were scored as labelled. The number of labelled cells for each point is expressed as a percentage of a total of 2,000 cells counted. ●——●, control $EB_4$ cells; ○——○, $10^{-5}$M MPS-treated $EB_4$ cells; ⊕, ⊖, represent the mean ± 1S.E. of 2 experiments each with duplicate observations; ●, ○, represent the mean ± range of duplicate observations from one experiment.

For calculation of growth fraction ($f_G$) see Appendix III(b), p. 185. The mean value for $t_{G1} + t_M + t_{G2}$ is calculated from the point of inflection of the 2 arms of the curve as shown.



in $t_{G1}$ by 7.6hr (substituting $t_{G2}$ and $t_M$ values from table 11, p. 140); again such an extrapolation is subjective as it depends on the drawing of curves by eye through data points. This increase in $t_{G1}$ is both qualitatively and quantitatively incompatible with $t_{G1}$ values obtained from PLM pulse-labelling curves (see tables 9 and 11, pp. 132 and 140) but this could be due to the different experimental designs, where the PLM pulse-labelling favours measurement of the fastest cycling population as opposed to the continuous-labelling technique which measures the entire proliferating population.

Effect of $10^{-5}$M MPS on population doubling times ($t_{DT}$) of $EB_4$ cells for $^3$H-TdR pulse-labelling and continuous-labelling experiments

Results for $t_{DT}$'s are present in table 12 (see p. 144). For both control and $10^{-5}$M MPS-treated $EB_4$ cells $t_{DT}$'s show values within the range previously recorded for the growth inhibitory response in Part II, Section 1.

Measurement of potential doubling time ($t_{C(pot)}$) and cell loss factor ($\phi$) from PLM pulse-labelling curves

$t_C$, obtained from PLM pulse-labelling (table 9, p. 132) is much shorter than $t_{DT}$, obtained from the increase in total cell concentration in the same experiments (table 12, p. 144), for both control and treated $EB_4$ cells. This discrepancy is expected and is due either to a decrease in growth fraction (Mendelsohn, 1962) or an increase in cell loss (Steel, 1968). The $f_G$ values obtained from PLM curves can be used to calculate $t_{C(pot)}$ (the doubling time of the population assuming no cell loss) and $\phi$ (the cell loss factor):

where $t_{C(pot)} = \frac{t_C}{f_G}$ (Mendelsohn, 1962)

and/...

TABLE 12 THE DOUBLING TIMES FOR CONTROL (WATER) AND $10^{-5}$M METHYLPREDNISOLONE (MPS)-TREATED $EB_4$ CELLS IN CELL CYCLE KINETIC EXPERIMENTS

| Experiment | Doubling time* (hr) for: | | Method of calculation |
|---|---|---|---|
| | control $EB_4$ cells | $10^{-5}$M MPS-treated $EB_4$ cells | |
| $^3$H-TdR pulse-LI and MI (figs. 50 and 51) | 35.9 ± 1.2 | 60.1 ± 4.5 | Mean ± 1S.E. over 12 days for 2 experiments each set up in duplicate |
| PLM curves after pulse-labelling with $^3$H-TdR (figs. 54 and 55) | 34.8 (range 34.3 - 35.4) | 52.7 (range 51.0 - 54.6) | Mean of duplicate tests over 48hr period used for determination of $t_C$ and its component phases |
| $^3$H-TdR continuous-LI and PLM curves (figs. 59 and 60) | 39.8 (range 35.4 - 44.1) | 54.9 (range 51.0 - 58.8) | Mean from 2 experiments over 48hr period used for determination of $t_{G2} + \frac{1}{2}t_M$ and $t_{G1} + t_M + t_{G2}$ |

*for calculation of doubling time see Appendix I(a), p. 181. Abbreviations used: $^3$H-TdR, $^3$H-thymidine; LI, labelling index; MI, mitotic index; PLM, percentage labelled mitoses; $t_C$, $t_{G2}$, $t_M$, $t_{G1}$, duration of the cell cycle time, G2, M, G1 respectively.

and $\phi = 1 - \frac{t_{C(pot)}}{t_{DT}}$ (Steel, 1968)

(these equations are also presented in context in Appendix III(c), p. 190).

The results for control and $10^{-5}$M MPS-treated cells are presented in table 13.

TABLE 13 ESTIMATION OF POTENTIAL DOUBLING TIME ($t_{C(pot)}$) AND CELL LOSS FACTOR ($\phi$) FROM PERCENTAGE OF LABELLED MITOSES (PLM) CURVES AFTER PULSE-LABELLING WITH $^3$H-THYMIDINE FOR BOTH CONTROL AND $10^{-5}$M METHYLPREDNISOLONE (MPS)-TREATED $EB_4$ CELLS

| Kinetic parameter | Values for: | |
|---|---|---|
| | Control $EB_4$ cells | $10^{-5}$M MPS-treated $EB_4$ cells |
| $t_{C(pot)}$ | 31.0hr | 45.8hr |
| $\phi$ | 0.11 | 0.13 |

The calculation of $t_{C(pot)}$ gives values similar to $t_{DT}$'s and $\phi$ values show a more or less equal cell loss factor of >10% for both control cells and treated cells. The $\phi$ value for control cells is surprisingly high bearing in mind the low background level of cell death observed in morphological studies.

COMMENT

These cell cycle kinetic studies on the growth inhibitory response of $EB_4$ cells to MPS have revealed that $10^{-5}$M MPS causes the following major effects:

(a) a stable reduction in the $^3$H-TdR pulse-LI

(b) a reduction in the MI

(c) conservation of $t_C$ and its components phases but with a decrease in $f_G$ when using the technique of PLM after pulse-labelling with $^3$H-TdR on a population of cells showing stable growth inhibition (i.e., after 6 days incubation with MPS)

(d) an apparent increase in $t_{G1}$ and a decrease in $f_G$ when using $^3$H-TdR continuous-labelling techniques on a population of cells showing stable growth inhibition.

These different results are discussed below, and from their interpretation a mechanism of action of MPS on the $EB_4$ cell cycle is proposed.

Stable reduction in pulse-LI (see fig. 50); reduction in MI (see fig. 51)

The reduction in pulse-LI (by 23%) in treated cells suggests that $10^{-5}$M MPS causes:

(a) either a decrease in $t_S$ - however this is not supported by PLM pulse-labelling data which suggests that $t_S$ values are conserved in treated cells (table 9, p. 132)

(b) or a decrease in $f_G$ - this is supported by calculations from both pulse-labelling and continuous-labelling PLM data where $f_G$ values of treated cells are lower than control cells by 27% and 14% respectively/...

respectively; this is also suggested by the observed reduction in MI for treated cells.

The stability of the reduction in LI implies that all cycling cells within the population remain potentially sensitive to the growth inhibitory effect of $10^{-5}$M MPS i.e., there cannot be a significant depletion by death of a sub-population of sensitive cells.

Conservation of $t_C$ and its phases as measured by PLM pulse-labelling technique (see figs. 54 and 55 and table 9, p. 132)

$t_{G2}$ values for control and treated cells as measured from PLM pulse-labelling curves were surprisingly long, indeed, about 3hr longer than when measured by the PLM continuous-labelling technique (cf. table 9, p. 132 with table 11, p. 140). This anomaly was also shown by Drewinko et al (1978) when comparing continuous- and pulse-labelling PLM techniques for measurement of cell cycle parameters on $T_1$ cells (a human lymphoma cell line derived from a patient with lymphocytic lymphoma). The effect could be caused by handling trauma inherent in the pulse-labelling technique which requires washing cells free of label, with ice-cold medium to prevent further incorporation, before assessing successive samples for PLM; therefore, for example, the cells experience a fall in temperature, a pH change during removal from the $CO_2$ atmosphere in the incubator, and the force of 3 consecutive centrifugations. Thus the metabolism of cells may be initially altered such that $G_2$ cells are temporarily 'blocked' or 'slowed down'. In addition, $t_{G2}$ elongation could be due to a $^3$H-TdR-induced radiation effect on $EB_4$ cells - this has been shown to result in a transient $G_2$ + M blockage in Chinese hamster fibroblasts with

$^3$H-TdR/...

$^{3}$H-TdR concentrations similar to that used for pulse-labelling (Ehmann et al, 1975).

Despite this anomaly, the results clearly show that $t_C$ and its component phases are similar for control and treated cells. This implies that in treated cultures, the passage of $EB_4$ cells around the cycle is unaffected once the decision to cycle is taken.

Reduction in $f_G$ as measured by PLM pulse-labelling and continuous-labelling techniques (see p. 136 and fig. 60 respectively)

The decrease in $f_G$ after treatment with $10^{-5}$M MPS as measured by PLM pulse-labelling (27%) and continuous-labelling (14%) techniques and the implication of a decrease in $f_G$ from pulse-LI and MI data, show the presence of an increased 'non-cycling' fraction in the treated cell population; moreover the pulse-LI data show that this is a stable increase.

The rapidity of the decrease in pulse-LI of treated cells (see fig. 50) suggests that this 'non-cycling' fraction of cells is pre-S, and the delay before the decrease in MI (fig. 51) excludes the possibility that these 'non-cycling' cells are pre-M; this therefore implies that the 'non-cycling' fraction is somewhere in $G_1$ and this is in general agreement with other studies both in vitro and in vivo which suggest that the growth inhibitory effect of glucocorticoid occurs due to an accumulation of cells in $G_1$ or a blockage of the transition of $G_1$ cells into S (Ernst and Killmann, 1970; Lampkin, McWilliams and Mauer, 1972; Mendelsohn, Multer and Bernheim, 1977; Norman and Thompson, 1977; Braunschweiger, Stragand and Schiffer, 1978).

The/...

The question now arises as to the fate of these 'non-cycling' or growth-inhibited cells and to this end I have proposed a cell cycle model for the mechanism of action of $10^{-5}$M MPS on $EB_4$ cells which is presented in fig. 61; it is based on the previously discussed model (fig. 1); 'non-cycling' cells may be considered as cells 'shunted' into $G_0$ or cells blocked in $G_1$. My results indicate that there are two possible pathways for the 'non-cycling' fraction induced by $10^{-5}$M MPS - these are illustrated in fig. 61(b):

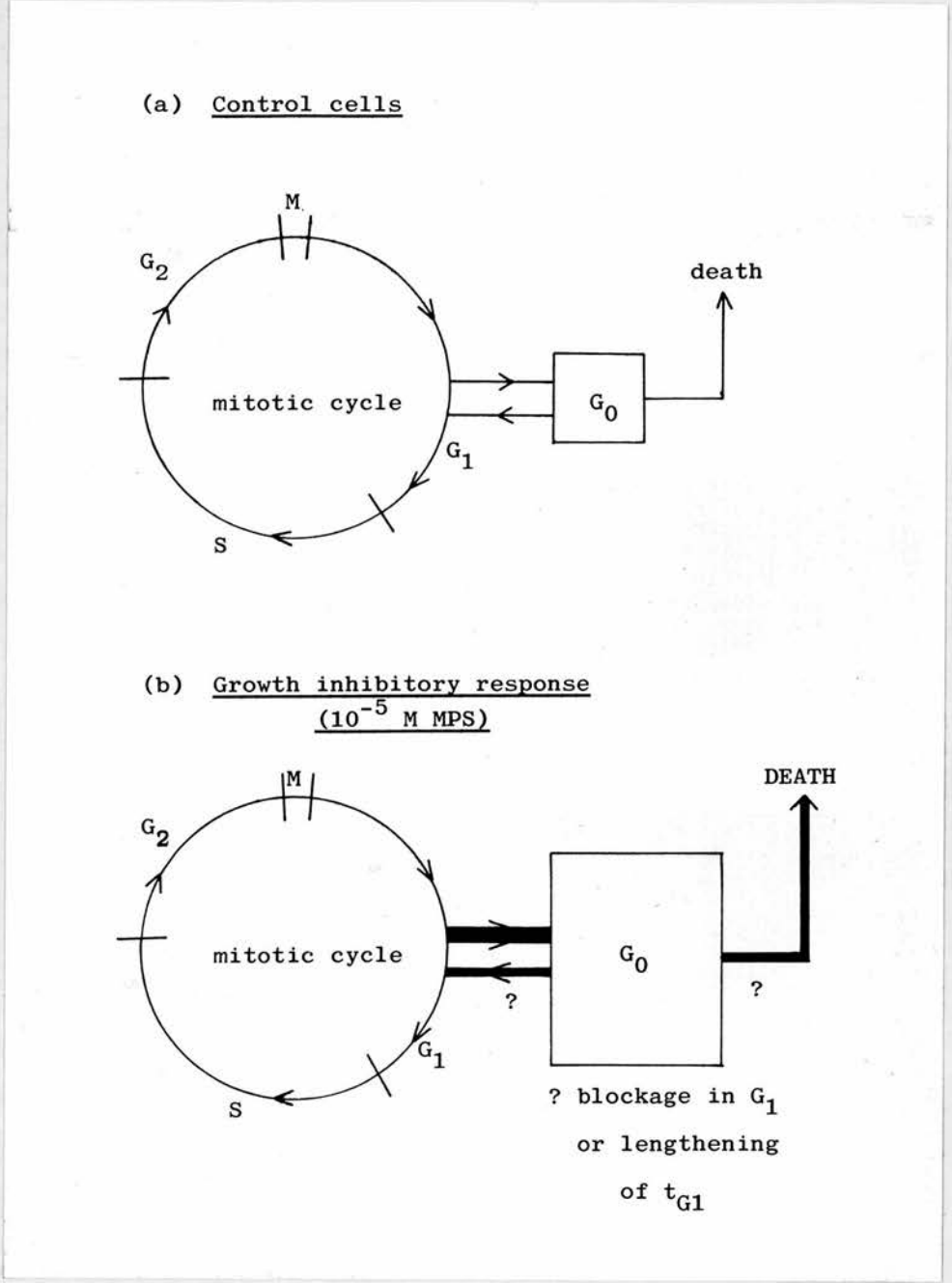
(a) 'non-cycling' cells may be committed to death - this is supported by morphological studies in the previous section where a small stable increase in cell death was observed in treated cultures (see fig. 43)

(b) 'non-cycling' cells may return to cycle after a delay in $G_1$ - this is supported by: (i) morphological studies in the previous section which suggest that some affected cells only show sub-lethal damage; (ii) continuous-LI curves (fig. 60) which suggest an apparent increase in $t_{G1}$ for treated cells; (iii) PLM pulse-labelling data which although suggesting that $t_C$ and its component phases are conserved for treated cells, do <u>not</u> preclude the possibility that a proportion of cells may return to cycle - PLM curves for treated cultures show damping out after 3 waves as compared to control cultures which show at least 4 waves (see figs. 54 and 55): this suggests a greater variation in $t_C$ or its phases in treated cultures which could be accounted for by an increased $t_{G1}$; moreover, as PLM curves tend to follow the fastest cycling population, a sub-population with an increased $t_{G1}$ may not necessarily be detected.

Unfortunately my results are not sufficiently quantitative to determine/...

Fig. 61

Cell cycle model of the proposed mechanism of action involved in $10^{-5}$ M methylprednisolone (MPS)-induced growth inhibition in $EB_4$ cells



determine more precisely the proportion of these 'non-cycling' cells committed to each pathway. However previous studies on glucocorticoid-induced growth inhibition in different cell types have suggested that both death of blocked cells (Norman, Harmon and Thompson, 1978) or return to cycle of blocked cells (Kollmorgen, 1969; Braunschweiger, Stragand and Schiffer, 1978) can be involved in the response.

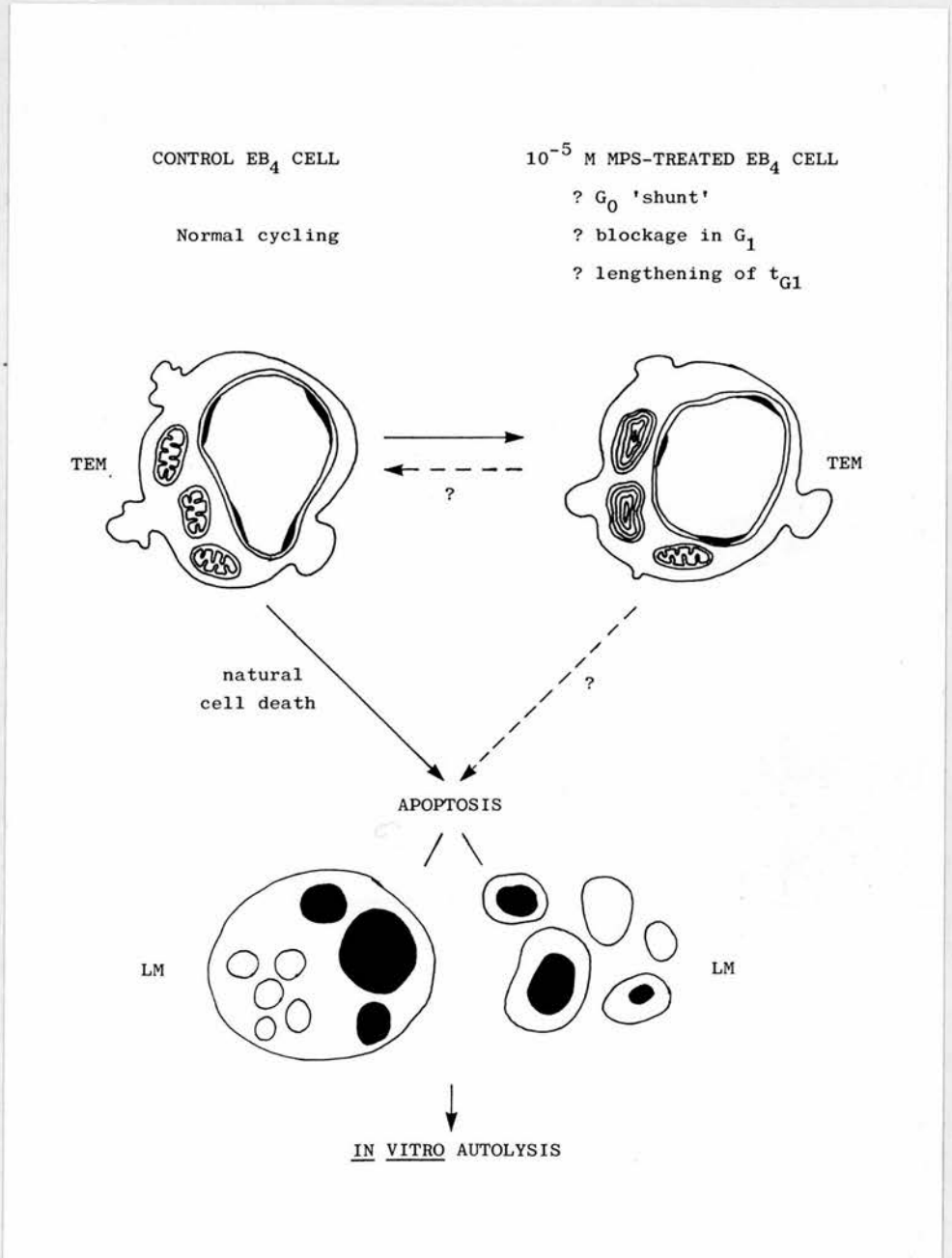
The question of whether blocked cells are committed to death or return to cycle could have important implications in the treatment of lymphoid tumours, which usually involves administration of glucocorticoids with other drugs which act on specific stages of the cell cycle. If blocked cells can return to cycle, then these cells will be protected against the effects of drugs which act on other phases (S, $G_2$ or M); if blocked cells die, then an additive effect should be achieved with other cell cycle active drugs - we have not as yet tested such possibilities.

Finally, the proposed cell cycle model for the action of $10^{-5}$M MPS on $EB_4$ cells has permitted further speculation on the observed morphological changes involved in growth inhibition and this is summarised schematically in fig. 62. Evidence remains insufficient to determine whether sub-lethally damaged cells can then return to cycle or whether they are irreversibly committed to death.

Fig. 62

Diagrammatic summary of the sequence of morphological changes occurring in $EB_4$ cells in vitro, with or without $10^{-5}$M methylprednisolone (MPS)

Interpretation is from transmission electron microscope (TEM) or light microscope (LM) studies.



PART II SUMMARY

SECTION 1 Exponentially growing cultures of $EB_4$ and $BLA_1$ cells showed a dose-dependent growth inhibitory response to MPS within the concentration range $10^{-7}$M - $10^{-4}$M MPS: there was an increase in the population $t_{DT}$ over 16 days incubation with MPS; there was also a small (5-10%) concomitant increase in cell death as assessed by nigrosine dye uptake which was dose-dependent and was not found until several days after treatment with MPS.

The $10^{-5}$M MPS growth inhibitory response in $EB_4$ cells was almost immediately reversible on removal of the glucocorticoid even after the cells had been incubated with MPS for 14 days.

SECTION 2 A study of the morphological changes involved in the growth inhibitory response of $EB_4$ cells to $10^{-5}$M MPS revealed that:

(a) with LM, there was a small increase in the number of apoptotic and autolytic cells in smears after 4 days treatment, and this was accompanied by a small increase in the percentage of cells taking up nigrosine. No other changes were evident in smears even after 16 days incubation with MPS.

(b) with TEM, in addition to apoptotic and autolytic changes, gross mitochondrial alterations were seen after 4 days treatment which included disorganisation of cristae and formation of myelin figures - these changes are indicative of an ongoing autophagic process.

SECTION 3/...

SECTION 3 Cell cycle kinetic analysis of the growth inhibitory response of $EB_4$ cells to $10^{-5}$M MPS revealed that inhibition is caused by a 'blockage' of a proportion of cells in $G_1$ (or $G_0$). The blocked cells may either be committed to death or may return to cycle with an apparently lengthened $t_{G1}$. This was proposed after studies had showed that $10^{-5}$M MPS causes:

(a) a stable reduction in the $^3$H-TdR pulse-LI

(b) a reduction in MI

(c) conservation of $t_C$ and its component phases, and a decrease in $f_G$ using the PLM $^3$H-TdR pulse-labelling technique

(d) an apparent increase in $t_{G1}$ and a decrease in $f_G$ when using $^3$H-TdR continuous-labelling techniques.

The implications of these results was briefly discussed.

## GENERAL DISCUSSION

In this thesis I have described the cytolethal and growth inhibitory responses of human lymphoid cell lines to methylprednisolone - these were analysed on a kinetic and morphological basis. It now remains to comment on the biochemical mechanisms which may be involved in these responses and the relationship between these responses and glucocorticoid-induced tumour regression *in vivo*.

Biochemical mechanisms involved in glucocorticoid-induced responses

Cytolethal response. There is much evidence to suggest that cytoplasmic receptors are involved in mediating the glucocorticoid-induced lethal effects via the classic gene-activation pathway (see fig. 3) in rodent lymphoid cells (Claman, 1972; Thompson and Lippman, 1974; Rosen and Milholland, 1975). However no proof exists as to whether this mechanism of action is involved in glucocorticoid-induced death in human lymphoid cells. The rôle played by receptors has therefore been investigated in our human lymphoid cell lines for the cytolethal response.

From receptor studies (Bird *et al*, 1975, 1977; Waddell, Bird and Currie, 1977a) it would appear that the $10^{-3}$M MPS-induced cytolethal response is *not* mediated by receptors as receptors become saturated around $10^{-7}$M MPS, a concentration 10,000 times lower than that which causes cell death. What mechanism of action may therefore be involved in the cytolethal response?

Evidence has been brought forward from other cell systems supporting/...

supporting the idea that the cell membrane may be the target for direct glucocorticoid interactions: e.g.,in cultured Novikoff rat hepatoma cells, inhibition of glucose transport takes place on immediate contact with glucocorticoid (Plagemann and Renner, 1972); surface alterations are evident in HeLa cells after addition of prednisolone (Fiskin and Melnykovych, 1971).

Two cell surface functions have therefore been investigated on our human lymphoid cell lines with $10^{-3}$M MPS:

(a) uptake of thymidine and uridine substrates - normal uptake of nucleosides consists of a 2-component system of both facilitated and simple diffusion pathways. $10^{-3}$M MPS appears to act at the cell surface by inhibiting facilitated diffusion uptake (Waddell, Bird and Currie, 1976, 1977b). In addition, this inhibition is instantaneous, which again supports non-involvement of specific cytoplasmic receptors in the mediation of the cytolethal response, as there is no time for altered gene activity to mediate this effect - this effect is minimal at concentrations $<10^{-3}$M MPS.

(b) concanavalin-A (con-A) agglutination - binding sites for con-A (a plant lectin) are present within the cell surface. Con-A agglutination of cells depends on the amount of con-A which binds to the sites and the distribution and mobility of the con-A binding sites. $10^{-3}$M MPS inhibits the rate of agglutination of cells probably by affecting the distribution of binding sites (Waddell et al, 1979) and this would indicate cell surface changes induced by the lethal concentration of MPS.

Thus the lethal $10^{-3}$M MPS response in our in vitro system would appear/...

appear to be associated with changes at the cell surface, and not with the cytoplasmic receptor gene-activation pathway.

A study of the sequence of morphological changes occurring after treatment with $10^{-3}$M MPS has also been of value in determining other mechanisms that may be involved in the glucocorticoid-induced cytolethal response in human lymphoid cells and one such possibility is the rôle played by intracellular ATP. Changes typical of apoptosis are observed within 1 - 2hr after treatment and this is followed by autolysis. It has been suggested that ATP plays an early primary rôle in cell death by coagulative necrosis (Trump and Arstila, 1971). However in human lymphoid cells it was found that intracellular ATP concentrations decrease after treatment with $10^{-3}$M MPS at the same rate as the number of cells excluding nigrosine (Waddell et al, 1979) i.e., the levels do not decrease until after apoptotic cells are morphologically detected in smears (see figs. 31 and 32). Thus, as apoptosis precedes ATP changes it can be concluded that ATP does not play a rôle in initiating the early morphological changes - this does not exclude the possibility that a decrease in the intracellular level of ATP is involved in the later appearance of autolysis.

Growth inhibitory response. Little is known about the biochemical mechanism of action of glucocorticoids involved in the growth inhibitory response of human lymphoid cells in vitro, but recent evidence would suggest that cytoplasmic receptor activity is involved (Chen, Aronow and Feldman, 1977; Norman and Thompson, 1977). Our studies on $EB_4$ cells (Waddell et al, 1979) would also suggest that the growth inhibitory response could be mediated via the receptors:

(a)/...

(a) the growth inhibitory response is apparent at about $10^{-7}$ - $10^{-6}$M MPS, the concentration range over which cytoplasmic glucocorticoid receptors become saturated (Bird et al, 1975)

(b) the relative growth inhibitory potencies of 3 glucocorticoids - triamcinolone acetonide (TA), MPS and cortisol (OHC) - could be correlated with their relative affinities for the receptors where TA > MPS = OHC

(c) recently we have isolated a sub-line of $EB_4$ cells resistant to the growth inhibitory effect of MPS with concentrations as high as $10^{-4}$M MPS - the resistant line consistently has less glucocorticoid receptors than the 'sensitive' line.

The reason for this loss of steroid binding capacity in resistant cells is not as yet known, but could be due to a decrease in the number or affinity of the specific glucocorticoid receptors, the presence of an inhibitor of glucocorticoid binding, or the destruction of receptors. This would agree with other isolated resistant cell line systems, namely cultured rodent lymphoid or fibroblastic cells, in which the resistant cells nearly always have quantitatively or qualitatively altered receptors or perhaps some deficiency in a later stage in the process of glucocorticoid hormone action (Pratt and Ishii, 1972; Rosenau et al, 1972; Kaiser, Milholland and Rosen, 1974; Sibley and Tomkins, 1974; Yamamoto, Stampfer and Tomkins, 1974).

The involvement of receptors in the growth inhibitory response implies a different mechanism of action of glucocorticoids from that involved in the cytolethal response - this is confirmed by studies which show that the cell line resistant to growth inhibition maintains its/...

its sensitivity to the $10^{-3}$M cytolethal response (Waddell et al, 1979). It remains to be determined whether the growth inhibitory response is also attributable to altered gene products i.e., whether the mechanism involved conforms to the classic receptor gene-mediated model for steroid hormone action.

Morphological studies on the growth inhibitory response can also be related to studies on intracellular ATP levels. Other than the small increase in cell death, the major morphological changes seen after treatment with $10^{-5}$M MPS are in the mitochondria which show autophagic activity, the result of sub-lethal damage. Autophagy, itself, is thought to be an energy requiring process which utilises ATP or other high energy intermediates (Trump and Arstila, 1971). In ATP studies, the ratio of ATP/$10^6$ nigrosine-excluding cells is found not to alter on exposure of cells to growth inhibitory concentrations of MPS; this suggests that ATP is in fact conserved and is consistent with ongoing autophagy.

Relationship between in vitro (cell line) and in vivo responses to glucocorticoid

Cytolethal response. The initial sequence of morphological changes in the $10^{-3}$M MPS-induced cytolethal response in vitro is consistent with apoptosis, the mode of cell death known to occur in the regression of certain tumours; hence this has provided a suitable model for studying the mechanisms involved in its initiation. However, two lines of evidence together would suggest that this high concentration effect has little relevance to human disease therapy:

(a) the highest therapeutic dose of steroid attainable in vivo to induce a remission response in leukaemia is about 1 - 5 x $10^{-5}$M

(b)/...

(b) the effect is not mediated by the classical receptor gene-activation pathway.

Growth inhibitory response. Growth inhibition in human lymphoid cell lines, which involves the blockage of cells in $G_1$ ($G_0$), appears to show more relevance to human disease therapy than the cytolethal effect:

(a) it occurs at concentrations of glucocorticoid attained in the therapy of patients with lymphoid neoplasia

(b) cytoplasmic glucocorticoid receptors are possibly involved in its initiation and much work has been done to suggest a rôle for receptors in the response in vivo.

With freshly obtained human leukaemic lymphoblasts Lippman et al (1973) found that the presence of specific cytoplasmic receptor molecules could be related to the responsiveness of the leukaemic patient in vivo, whereas normal lymphocyte controls which are insensitive to glucocorticoids showed no cytoplasmic receptor activity. In patients who originally responded to drug therapy which included glucocorticoids, glucocorticoid receptor activity was present; however when these patients developed resistance to glucocorticoid therapy no glucocorticoid receptor activity was evident. It was also shown that $^3$H-thymidine uptake was not inhibited by glucocorticoids in leukaemic blast cells which lacked receptor activity.

This result was confirmed in a later study by Lippman, Perry and Thompson (1975) on cells from patients with acute myeloblastic leukaemia (AML) where 13 out of 16 patients lacked glucocorticoid receptor/...

receptor activity. This correlates with the known response of AML patients to treatment with glucocorticoids, where remission is only occasionally achieved.

Gailani et al (1973) also studied glucocorticoid receptor activity in cells obtained from patients with ALL, AML and chronic lymphocytic leukaemia (CLL). Again, the presence of glucocorticoid receptors could be related to the frequent clinical response of these leukaemias to glucocorticoid treatment.

Yarbro et al (1977) have also shown that lymphoblasts from ALL patients can be divided into prognostic groups based on glucocorticoid receptor content and cell surface immunological markers. Lymphoblasts in the patients studied were characterised as either T cells or null cells (no B cells were recorded in this study) and it was found that null cells contained three times as many receptors per cell as T cells. This correlates with clinical evidence which suggests that null-cell leukaemias respond more favourably to therapy than T cell leukaemias.

However, whilst these four studies seem to suggest that it is necessary for leukaemic cells to have steroid receptors for the patient to show a therapeutic response, more recent studies have questioned the prognostic value of receptors (Duval and Homo, 1978; Iacobelli et al, 1978). Iacobelli et al (1978) in fact showed that freshly isolated leukocytes from a patient with ALL contained receptors and responded to the inhibitory effects of glucocorticoids in vitro, yet the patient responded to glucocorticoid therapy with an increase in the white blood cell count.

This lack of a general rôle for receptor assays emerging from recent/...

recent studies could be explained from my cell cycle kinetic results where the stability of the growth inhibitory response implies that all cycling cells within the population remain potentially sensitive to the growth inhibitory effect of $10^{-5}$M MPS - thus although cells possess receptors they may not necessarily respond.

Whilst cell death, and possibly a lengthening of $t_{G1}$ play a small rôle in the growth inhibitory response, it is difficult to see how these low level effects could account for the marked decrease in blood blast cell count (Henderson, 1969; Lampkin, Nagao and Mauer, 1969; Ernst and Killmann, 1970) and hyperuricaemia (Sandberg, Cartwright and Wintrobe, 1956; Wolff et al, 1967) seen in some leukaemic patients after glucocorticoid therapy.

How, therefore, can the observed in vitro responses be related to the in vivo clinical remission response?
There are three possible explanations:

(a) the cell lines have been maintained in culture for many years and they may have changed in their sensitivity to glucocorticoids. In this respect, freshly isolated human neoplastic lymphoid cells treated with glucocorticoids might have provided an in vitro system more directly comparable to in vivo therapy - however, we did not use this system for reasons already detailed in the General Introduction

(b) glucocorticoids in in vivo therapy may alter lymphoid tumour cells such that they are 'recognised' and destroyed by phagocytic cells (i.e., host defence mechanisms)

(c) the glucocorticoid response may be enhanced in vivo by interaction of glucocorticoid with other circulating factors.

Such explanations are undoubtedly oversimplified as glucocorticoids have/...

have also been shown to exert other effects which may be responsible for life-support in patients undergoing chemotherapy:

(a) immunosuppressive and anti-inflammatory effects (see review by Fauci, Dale and Balow, 1976) - such effects include: changes in the function of lymphocytes, leukocytes and monocytes; a marked transient lymphocytopoenia and monocytopoenia which is caused by an apparent redistribution of the intravascular recirculating lymphocyte pool into extravascular lymphoid compartments (the spleen, lymph nodes, thoracic duct and bone marrow)

(b) a potentiation effect with other drugs used in combination therapy. Long-term treatment of ALL is only successful with drug combination therapy and not with glucocorticoid therapy alone. Glucocorticoids are usually combined with drugs effective in other parts of the cell cycle e.g., vincristine, which arrests cells in mitosis and blocks entry of resting cells into the mitotic cycle (Lampkin, McWilliams and Mauer, 1972) or methotrexate which inhibits DNA synthesis (Ernst and Killmann, 1970; Lampkin, McWilliams and Mauer, 1972). It is possible that these drugs in combination may either exert their effects independently, or perhaps more importantly, may act synergistically with glucocorticoids to cause cell kill - indeed in vitro studies on human lymphoid cell lines have shown that glucocorticoids in combination with vincristine (Rosner et al, 1975; Norman, Harmon and Thompson, 1978) and methotrexate (Norman, Harmon and Thompson, 1978) show synergistic activity. However in the same context, Norman, Harmon and Thompson (1978) have also shown that prednisolone combined with 6-mercaptopurine, which arrests cells in S-phase, gives an effect which is less than additive - thus not all drug combinations may show potentiation in in vivo therapy

(c) induction of cell differentiation (Weintraub, 1972). In vivo, lymphoid cells are a heirarchy of stem cells, differentiating cells and/...

and mature cells, whose proliferation and differentiation are under homeostatic control - this involves positive or negative feedback control mediated by cellular or humoral events (Cline and Golde, 1979). In lymphoid tumours, normal homeostasis is altered due to an accummulation of malignant cells which fail to differentiate fully and which may produce humoral factors which suppress the growth of normal haematopoietic cells (Cline and Golde, 1979) - thus a stimulation of cell differentiation might have an important significance in therapy.

Thus the mechanism of action of glucocorticoids in vivo may be far more complex than can be analysed from studies on human lymphoid cell lines.

Future developments

From these studies, two interesting areas have emerged for further investigation.

Firstly, it is necessary to develop a more sophisticated in vitro technique such that we can predict the in vivo response of patients with lymphoid neoplasia to glucocorticoid therapy. Recently, for example, measurement of the ability of bone marrow cells from patients with myeloma and carcinoma to form colonies in in vitro agar culture after exposure to certain chemotherapeutic agents, has shown promise as a predictive assay for the in vivo response (Salmon et al, 1978).

Secondly, it is necessary to determine more precisely the rôle played by receptors and the gene activation pathway in glucocorticoid-induced growth inhibition in human lymphoid cells. The isolation of sub-lines/...

sub-lines resistant to the growth inhibitory effect has provided a tool for such a study, where analysis of glucocorticoid-induced effects by such means as fusion of sensitive and resistant cells may give some indication of the mechanisms involved.

Hopefully such future studies may enlarge our understanding of the mechanisms involved in glucocorticoid-induced tumour regression in vivo such that chemotherapeutic régimes which include glucocorticoids may be employed on a more rational basis.

REFERENCES

ADOLPHE, M. and LECHAT, P. (1974). Action of a steroid anti-inflammatory agent (methylprednisolone) on the cell cycle: study on synchronized cells. Biomedicine, 20, 46-53.

AHERNE, W.A., CAMPLEJOHN, R.S. and WRIGHT, N.A. (1977). In An Introduction to Cell Population Kinetics, Edward Arnold (Publishers) Ltd., London, pp. 27-43.

BALLARD, K.J. and HOLT, S.J. (1968). Cytological and cytochemical studies on cell death and digestion in the foetal rat foot: the role of macrophages and hydrolytic enzymes. J. Cell Sci., 3, 245-262.

BARAN, D.T., LICHTMAN, M.A. and PECK, W.A. (1972). Alpha-amino-isobutyric acid transport in human leukemic lymphocytes: in vitro characteristics and inhibition by cortisol and cycloheximide. J. Clin. Invest., 51, 2182-2189.

BARRETT, J.C. (1966). A mathematical model of the mitotic cycle and its application to the interpretation of percentage labelled mitoses data. J. Natl. Cancer Inst., 37, 443-450.

BASERGA, R. and WIEBEL, F. (1969). The cell cycle of mammalian cells. Int. Rev. Exp. Pathol., 7, 1-30.

BAULIEU, E-E. (1975). Some aspects of the mechanism of action of steroid hormones. Mol. Cell. Biochem., 7, 157-174.

BEDFORD, J.S., MITCHELL, J.B., GRIGGS, H.G. and BENDER, M.A. (1975). Cell killing by gamma rays and beta particles from tritiated water and incorporated tritiated thymidine. Radiat. Res., 63, 531-543.

BELL, P.A. and BORTHWICK, N.M. (1976). Glucocorticoid effects on DNA-dependent RNA polymerase activity in rat thymus cells. J. Steroid Biochem., 7, 1147-1150.

BERARD/...

BERARD, C.W., GALLO, R.C., JAFFE, E.S., GREEN, I. and DeVITA, V.T.(JR) (1976). Current concepts of leukemia and lymphoma: etiology, pathogenesis, and therapy. Ann. Intern. Med., 85, 351-366.

BIRD, C.C., ROBERTSON, A.M.G., READ, J. and CURRIE, A.R. (1977). Cytolethal effects of glucocorticoids in human lymphoblastoid cell lines. J. Pathol., 123, 145-156.

BIRD, C.C., WADDELL, A.W., ROBERTSON, A.M.G., CURRIE, A.R., STEEL, C.M. and EVANS, J. (1975). Cytoplasmic receptor levels and glucocorticoid response in human lymphoblastiod cell lines. Br. J. Cancer, 32, 700-707.

BLACK, L. and BERENBAUM, M.C. (1964). Factors affecting the dye exclusion test for cell viability. Exp. Cell Res., 35, 9-13.

BRAUNSCHWEIGER, P.G., STRAGAND, J.J. and SCHIFFER, L.M. (1978). Effect of methylprednisolone on cell proliferation in C3H/HeJ spontaneous mammary tumors. Cancer Res., 38, 4510-4514.

BREARLEY, R.L., LISTER, T.A., WHITEHOUSE, J.M.A. and STANSFELD, A.G. (1977). Burkitt's lymphoma in British adults: clinical features and response to chemotherapy. Br. J. Cancer, 35, 484-487.

BURTON, A.F., STORR, J.M. and DUNN, W.L. (1967). Cytolytic action of corticosteroids on thymus and lymphoma cells in vitro. Can. J. Biochem., 45, 289-297.

BURTON, R.M. and WESTPHAL, U. (1972). Steroid hormone-binding proteins in blood plasma. Metabolism, 21, 253-276.

CHEN, T.L., ARONOW, L. and FELDMAN, D. (1977). Glucocorticoid receptors and inhibition of bone cell growth in primary culture. Endocrinology, 100, 619-628.

CLAESSON, M.H. (1969). Quantitative studies on the normal decay of lymphocytes in the thymolymphatic system. Scand. J. Haematol., 6, 87-92.

CLAMAN/...

CLAMAN, H.N. (1972). Corticosteroids and lymphoid cells. N. Engl. J. Med., 287, 388-397.

CLEAVER, J.E., THOMAS, G.H. and BURKI, H.J. (1972). Biological damage from intranuclear tritium: DNA strand breaks and their repair. Science, 177, 996-998.

CLINE, M.J. and GOLDE, D.W. (1979). Cellular interactions in haematopoiesis. Nature, 277, 177-181.

CRAWFORD, A.M., KERR, J.F.R. and CURRIE, A.R. (1972). The relationship of acute mesodermal cell death to the teratogenic effects of 7-OHM-12-MBA in the foetal rat. Br. J. Cancer, 26, 498-503.

DeVITA, V.T. (1973). Combined drug treatment of Hodgkin's disease: remission induction, remission duration, and survival; an appraisal. Natl. Cancer Inst. Monogr., 36, 373-379.

DOUGHERTY, T.F. and WHITE, A. (1945). Functional alterations in lymphoid tissue induced by adrenal cortical secretion. Am. J. Anat., 77, 81-116.

DREWINKO, B., BOBO, B., ROPER, P.R., MALAHY, M.A., BARLOGIE, B. and JANSSON, B. (1978). Analysis of the growth kinetics of a human lymphoma cell line. Cell Tissue Kinet., 11, 177-191.

DUVAL, D. and HOMO, F. (1978). Prognostic value of steroid receptor determination in leukemia. Cancer Res., 38, 4263-4267.

EHMANN, U.K., WILLIAMS, J.R., NAGLE, W.A., BROWN, J.A., BELLI, J.A. and LETT, J.T. (1975). Perturbations in cell cycle progression from radioactive DNA precursors. Nature, 258, 633-636.

ERNST, P. and KILLMANN, S-A.,(1970). Perturbation of generation cycle of human leukemic blast cells by cytostatic therapy in vivo: effect of corticosteroids. Blood, 36, 689-696.

EZDINLI/...

EZDINLI, E.Z., STUTZMAN, L., AUNGST, C.W. and FIRAT, D. (1969). Corticosteroid therapy for lymphomas and chronic lymphocytic leukemia. Cancer, 23, 900-909.

FALLON, J.F. and CAMERON, J.A. (1977). Interdigital cell death during limb development of the turtle and lizard with an interpretation of evolutionary significance. J. Embryol. Exp. Morphol., 40, 285-289.

FAUCI, A.S., DALE, D.C. and BALOW, J.E. (1976). Glucocorticosteroid therapy: mechanisms of action and clinical considerations. Ann. Intern. Med., 84, 304-315.

FISKIN, A.M. and MELNYKOVYCH, G. (1971). Prednisolone induced surface alterations in HeLa cells. Exp. Cell Res., 66, 483-486.

GAILANI, S., MINOWADA, J., SILVERNAIL, P., NUSSBAUM, A., KAISER, N., ROSEN, F. and SHIMAOKA, K. (1973). Specific glucocorticoid binding in human hemopoietic cell lines and neoplastic tissue. Cancer Res., 33, 2653-2657.

GRASSO, R.J., JOHNSON, C.E., BOLER, R.K. and MOORE, N.A. (1977). Combined growth-inhibitory responses and ultrastructural alterations produced by 1, 3-bis (2-chloroethyl)-1-nitrosourea and dexamethasone in rat glioma cell cultures. Cancer Res., 37, 585-594.

HABESHAW, J.A., MACAULAY, R.A.A. and STUART, A.E. (1977). Correlation of surface receptors with histological appearance in 29 cases of non-Hodgkin lymphoma. Br. J. Cancer, 35, 858-867.

HARRIS, A.W. (1970). Differentiated functions expressed by cultured mouse lymphoma cells. I. Specificity and kinetics of cell responses to corticosteroids. Exp. Cell Res., 60, 341-353.

HAWKINS/...

HAWKINS, H.K., ERICSSON, J.L.E., BIBERFELD, P. and TRUMP, B.F. (1972). Lysosome and phagosome stability in lethal cell injury. Morphologic tracer studies in cell injury due to inhibition of energy metabolism, immune cytolysis and photosensitization. Am. J. Pathol., 68, 255-288.

HENDERSON, E.S. (1969). Treatment of acute leukaemia. Semin. Hematol., 6, 271-319.

HIGGINS, S.J. and GEHRING, U. (1978). Molecular mechanisms of steroid hormone action. Adv. Cancer Res., 28, 313-397.

HILL, B.T. (1976). Biochemistry of the cell cycle. In Scientific Foundations of Oncology, edited by T. Symington and R.L. Carter, William Heinemann Medical Books Ltd., London, pp. 63-72.

HIRSHAUT, Y., WEISS, G.H. and PERRY, S. (1969). The use of long-term human leukocyte cell cultures as models for the study of anti-leukemic agents. Cancer Res., 29, 1732-1740.

HOWARD, A. and PELC, S.R. (1953). Synthesis of desoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity, Suppl. 6, 261-273.

IACOBELLI, S., RANELLETTI, F.O., LONGO, P., RICCARDI, R. and MASTRANGELO, R. (1978). Discrepancies between in vivo and in vitro effects of glucocorticoids in myelomonocytic leukemic cells with steroid receptors. Cancer Res., 38, 4257-4262.

JENNINGS, R.B., GANOTE, C.E. and REIMER, K.A. (1975). Ischemic tissue injury. Am. J. Pathol., 81, 179-198.

JENSEN, E.M., KOROL, W., DITTMAR, S.L. and MEDREK, T.J. (1967). Virus containing lymphocyte cultures from cancer patients. J. Natl. Cancer Inst., 39, 745-754.

JONES/...

JONES, K.L., ANDERSON, N.S., III and ADDISON, J. (1978). Glucocorticoid-induced growth inhibition of cells from a human lung alveolar cell carcinoma. Cancer Res., 38, 1688-1693.

KAISER, N., MILHOLLAND, R.J. and ROSEN, F. (1974). Glucocorticoid receptors and mechanism of resistence in the cortisol-sensitive and -resistant lines of lymphosarcoma P1798. Cancer Res., 34, 621-626.

KALTENBACH, J.P., KALTENBACH, M.H. and LYONS, W.B. (1958). Nigrosin as a dye for differentiating live and dead ascites cells. Exp. Cell Res., 15, 112-117.

KERR, J.F.R. (1971). Shrinkage necrosis: a distinct mode of cellular death. J. Pathol., 105, 13-20.

KERR, J.F.R. (1972). Some lysosome functions in liver cells reacting to sublethal injury. In Lysosomes in Biology and Pathology, edited by J.T. Dingle and H.B. Fell, North-Holland, Amsterdam, 3, pp. 365-394.

KERR, J.F.R. and SEARLE, J. (1972a). A suggested explanation for the paradoxically slow growth rate of basal-cell carcinomas that contain numerous mitotic figures. J. Pathol., 107, 41-44.

KERR, J.F.R. and SEARLE, J. (1972b). The digestion of cellular fragments within phagolysosomes in carcinoma cells. J. Pathol., 108, 55-58.

KERR, J.F.R. and SEARLE, J. (1973). Deletion of cells by apoptosis during castration-induced involution of the rat prostate. Virchows Archiv. Abt. B Zellpath., 13, 87-102.

KERR, J.F.R., HARMON, B. and SEARLE, J. (1974). An electron-microscope study of cell deletion in the anuran tadpole tail during spontaneous metamorphosis with special reference to apoptosis of striated muscle fibres. J. Cell Sci., 14, 571-585.

KERR/...

KERR, J.F.R., WYLLIE, A.H. and CURRIE, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer, 26, 239-257.

KOLLMORGEN, G.M. (1969). The response of HeLa cells to irradiation and hydrocortisone. Natl. Cancer Inst. Monogr., 30, 157-167.

LAIHO, K.U. and TRUMP, B.F. (1974). Relationship of ionic, water and cell volume changes in cellular injury of Ehrlich ascites tumor cells. Lab. Invest., 31, 207-215.

LAJTHA, L.G. (1963). On the concept of the cell cycle. J. Cell Comp. Physiol., 62 (Suppl. 1), 143-145.

LAMERTON, L.F. (1976). Cell population kinetics in normal and malignant tissues. In Scientific Foundations of Oncology, edited by T. Symington and R.L. Carter, William Heinemann Medical Books Ltd., London, pp. 119-125.

LAMPKIN, B.C., MCWILLIAMS, N.B. and MAUER, A.M. (1972). Cell kinetics and chemotherapy in acute leukemia. Semin. Hematol., 9, 211-223.

LAMPKIN, B.C., NAGAO, T. and MAUER, A.M. (1969). Drug effect in acute leukemia. J. Clin. Invest., 48, 1124-1130.

LAMPKIN, B.C., NAGAO, T. and MAUER, A.M. (1971). Synchronization and recruitment in acute leukemia. J. Clin. Invest., 50, 2204-2214.

LEUNG, K. and MUNCK, A. (1975). Long-term incubation of rat thymus cells: cytolytic actions of glucocorticoids in vitro. Endocrinology, 97, 744-748.

LEVENTHAL, B.G. and KONIOR, G.S. (1976). Leukemia: a critical review. Semin. Oncol., 3, 319-325.

LIPPMAN, M.E., PERRY, S. and THOMPSON, E.B. (1975). Glucocorticoid binding proteins in myeloblasts of acute myelogenous leukemia. Am. J. Med., 59, 224-227.

LIPPMAN/...

LIPPMAN, M.E., HALTERMAN, R.H., LEVENTHAL, B.G., PERRY, S. and THOMPSON, E.B. (1973). Glucocorticoid-binding proteins in human acute lymphoblastic leukemic blast cells. J. Clin. Invest., 52, 1715-1725.

MCDOWELL, E.M. (1972a). Light- and electron-microscope studies of the rat kidney after administration of inhibitors of the citric acid cycle in vivo: changes in the proximal convoluted tubule during fluorocitrate poisoning. J. Pathol., 108, 303-318.

MCDOWELL, E.M. (1972b). Light and electron microscopic studies of the rat kidney after administration of inhibitors of the citric acid cycle in vivo. I. Effects of sodium fluoroacetate on the proximal convoluted tubule. Am. J. Pathol., 66, 513-542.

MCLEAN, A.E.M., MCLEAN, E. and JUDAH, J.D. (1965). Cellular necrosis in the liver induced and modified by drugs. Int. Rev. Exp. Pathol., 4, 127-157.

MAJNO, G. (1964). Interactions between dead cells and living tissue. In Ciba Foundation Symposium on Cellular Injury, edited by A.V.S. de Reuck and J. Knight, Churchill, London, pp. 87-105.

MAKMAN, M.H., NAKAGAWA, S. and WHITE, A. (1967). Studies on the mode of action of adrenal steroids on lymphocytes. Recent Prog. Horm. Res., 23, 195-227.

MAUER, A.M., SIMONE, J.V. and PRATT, C.B. (1977). Current progress in the treatment of the child with cancer. J. Pediatr., 91, 523-539.

MEDZIHRADSKY, F. and MARKS, M.J. (1975). Measures of viability in isolated cells. Biochem. Med., 13, 164-177.

MENDELSOHN, J., MULTER, M.M. and BERNHEIM, J.L. (1977). Inhibition of human lymphocyte stimulation by steroid hormones: cytokinetic mechanisms. Clin. Exp. Immunol., 27, 127-134.

MENDELSOHN/...

MENDELSOHN, M.L. (1962). Autoradiographic analysis of cell proliferation in spontaneous breast cancer of C3H mouse. III. The growth fraction. J. Natl. Cancer Inst., 28, 1015-1029.

MITCHISON, J.M. (1971). In The Biology of the Cell Cycle, Cambridge University Press, pp. 21-22 and 26-28.

MOSHER, K.M., YOUNG, D.A. and MUNCK, A. (1971). Evidence for irreversible, actinomycin D-sensitive, and temperature-sensitive steps following binding of cortisol to glucocorticoid receptors and preceding effects on glucose metabolism in rat thymus cells. J. Biol. Chem., 246, 654-659.

MYERS, D.K. and De WOLFE-SLADE, D. (1964). Some effects of cationic condensing agents on rat thymocytes. Exp. Cell Res., 33, 344-349.

NACHT, S. and GARZÓN, P. (1974). Effects of corticosteroids on connective tissue and fibroblasts. Adv. Steroid Biochem. Pharmacol., 4, 157-187.

NILSSON, K. (1971). Characteristics of established myeloma and lymphoblastoid cell lines derived from an E myeloma patient: a comparative study. Int. J. Cancer, 7, 380-396.

NILSSON, K. and PONTÉN, J. (1975). Classification and biological nature of established human hematopoietic cell lines. Int. J. Cancer, 15, 321-341.

NORMAN, M.R. and THOMPSON, E.B. (1977). Characterization of a glucocorticoid-sensitive human lymphoid cell line. Cancer Res., 37, 3785-3791.

NORMAN, M.R., HARMON, J.M. and THOMPSON, E.B. (1978). Use of a human lymphoid cell line to evaluate interactions between prednisolone and other chemotherapeutic agents. Cancer Res., 38, 4273-4278.

PARKER/...

PARKER, J.W., TAYLOR, C.R., PATTENGALE, P.K., ROYSTON, I., TINDLE, B.H., CAIN, M.J. and LUKES, R.J. (1978). Morphologic and cytochemical comparison of human lymphoblastoid T-cell and B-cell lines: light and electron microscopy. J. Natl. Cancer Inst., 60, 59-68.

PHILLIPS, H.J. and TERRYBERRY, J.E. (1957). Counting actively metabolising tissue cultured cells. Exp. Cell Res., 13, 341-347.

PLAGEMANN, P.G.W. and RENNER, E.D. (1972). Glucocorticoids: competitive inhibition of glucose transport. Biochem. Biophys. Res. Commun., 46, 816-823.

PONEC, M., De HAAS, C., BACHRA, B.N. and POLANO, M.K. (1977). Effects of glucocorticosteroids on primary human skin fibroblasts. I. Inhibition of the proliferation of cultured primary human skin and mouse L929 fibroblasts. Arch. Dermatol. Res., 259, 117-123.

POTTEN, C.S., AL-BARWARI, S.E. and SEARLE, J. (1978). Differential radiation response amongst proliferating epithelial cells. Cell Tissue Kinet., 11, 149-160.

PRATT, W.B. and ARONOW, L. (1966). The effect of glucocorticoids on protein and nucleic acid synthesis in mouse fibroblasts growing in vitro. J. Biol. Chem., 241, 5244-5250.

PRATT, W.B. and ISHII, D.N. (1972). Specific binding of glucocorticoids in vitro in the soluble fraction of mouse fibroblasts. Biochemistry, 11, 1401-1410.

PULVERTAFT, R.J.V. (1965). A study of malignant tumours in Nigeria by short-term tissue culture. J. Clin. Pathol., 18, 261-273.

QUASTLER, H. and SHERMAN, F.G. (1959). Cell population kinetics in the intestinal epithelium of the mouse. Exp. Cell Res., 17, 420-438.

ROPER/...

ROPER, P.R. and DREWINKO, B. (1976). Comparison of *in vitro* methods to determine drug-induced cell lethality. Cancer Res., 36, 2182-2188.

ROSEN, F. and MILHOLLAND, R.J. (1975). Mechanism of action of glucocorticoids. In Handbook of Experimental Pharmacology, edited by A.C. Sartorelli and D.G. Johns, XXXVIII/2, pp. 85-103.

ROSENAU, W., BAXTER, J.D., ROUSSEAU, G.G. and TOMKINS, G.M. (1972). Mechanism of resistance to steroids: glucocorticoid receptor defect in lymphoma cells. Nature, New Biol., 237, 20-23.

ROSNER, F., HIRSHAUT, Y., GRÜNWALD, H.W. and DIETRICH, M. (1975). *In vitro* combination chemotherapy demonstrating potentiation of vincristine cytotoxicity by prednisolone. Cancer Res., 35, 700-705.

RUSSELL, S.W., ROSENAU, W. and LEE, J.C. (1972). Cytolysis induced by human lymphotoxin. Cinemicrographic and electron microscopic observations. Am. J. Pathol., 69, 103-118.

SALADINO, A.J. and TRUMP, B.F. (1968). Ion movements in cell injury. Effects of inhibition of respiration and glycolysis on the ultrastructure and function of the epithelial cells of the toad bladder. Am. J. Pathol., 52, 737-776.

SALMON, S.E., HAMBURGER, A.W., SOEHNLEN, B., DURIE, B.G.M., ALBERTS, D.S. and MOON, T.E. (1978). Quantitation of differential sensitivity of human-tumor stem cells to anticancer drugs. N. Engl. J. Med., 298, 1321-1327.

SANDBERG, A.A., CARTWRIGHT, G.E. and WINTROBE, M.M. (1956). Studies on leukaemia. I. Uric acid excretion. Blood, 11, 154-166.

SCHEIN, P.S., CHABNER, B.A., CANELLOS, G.P., YOUNG, R.C., BERARD, C. and DeVITA, V.T. (1975). Results of combination of chemotherapy of non-Hodgkin's lymphoma. Br. J. Cancer, 31, Suppl. II, 465-473.

SCHREK/...

SCHREK, R. (1936). A method for counting the viable cells in normal and in malignant cell suspensions. Am. J. Cancer, 28, 389-392.

SCHUTZ, G., KILLEWICH, L., CHEN, G. and FEIGELSON, P. (1975). Control of the mRNA for hepatic tryptophan oxygenase during hormonal and substrate induction. Proc. Natl. Acad. Sci., U.S.A. 72, 1017-1020.

SEARLE, J., COLLINS, D.J., HARMON, B. and KERR, J.F.R. (1973). The spontaneous occurrence of apoptosis in squamous carcinomas of the uterine cervix. Pathology, 5, 163-169.

SEARLE, J., LAWSON, T.A., ABBOTT, P.J., HARMON, B. and KERR, J.F.R. (1975). An electron-microscope study of the mode of cell death induced by cancer-chemotherapeutic agents in populations of proliferating normal and neoplastic cells. J. Pathol., 116, 129-138.

SIBLEY, C.H. and TOMKINS, G.M. (1974). Mechanisms of steroid resistance. Cell, 2, 221-227.

SIMONE, J.V., AUR, R.J.A., HUSTU, H.O., VERZOSA, M.S. and PINKEL, D. (1978). Three to ten years after cessation of therapy in children with leukemia. Cancer, 42, 839-844.

STEEL, C.M. (1972). Human lymphoblastoid cell lines. III. Co-cultivation technique for establishment of new lines. J. Natl. Cancer Inst., 48, 623-628.

STEEL, C.M. and EDMOND, E. (1971). Human lymphoblastoid cell lines. I. Culture methods and examination for Epstein-Barr virus. J. Natl. Cancer Inst., 47, 1193-1201.

STEEL, G.G. (1967). Cell loss as a factor in the growth rate of human tumours. Eur. J. Cancer, 3, 381-387.

STEEL, G.G. (1968). Cell loss from experimental tumours. Cell Tissue Kinet., 1, 193-207.

STEEL/...

STEEL, G.G. (1977). In Growth Kinetics of Tumours, Clarendon Press, Oxford, pp. 56-143.

STEEL, G.G. and HANES, S. (1971). The technique of labelled mitoses: analysis by automatic curve-fitting. Cell Tissue Kinet., 4, 93-105.

STORY, M.T. and MELNYKOVYCH, G. (1973). Growth inhibition of mouse lymphoma cells, L5178Y, in vitro, by glucocorticoids. Alterations in choline metabolism. Exp. Cell Res., 77, 437-449.

TANNOCK, I.F. (1968). The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. Br. J. Cancer, 22, 258-273.

THOMLINSON, R.H. and GRAY, L.H. (1955). The histological structure of some human lung cancers and the possible implications for radiotherapy. Br. J. Cancer, 9, 539-549.

THOMPSON, E.B. and LIPPMAN, M.E. (1974). Mechanism of action of glucocorticoids. Metabolism, 23, 159-202.

TROWELL, O.A. (1966). Ultrastructural changes in lymphocytes exposed to noxious agents in vitro. Q. J. Exp. Physiol., 51, 207-220.

TRUMP, B.F. and ARSTILA, A.U. (1971). Cell injury and cell death. In Principles of Pathobiology, edited by M.F. La Via and R.B. Hill, Jr, Oxford University Press, New York, London and Toronto, pp. 9-95.

TRUMP, B.F. and ERICSSON, J.L.E. (1965). Some ultrastructural and biochemical consequences of cell injury. In The Inflammatory Process, edited by B.W. Zweifach, L. Grant and R.T. McCluskey, Academic Press, New York and London, pp. 35-120.

TRUMP, B.F. and GINN, F.L. (1969). The pathogenesis of subcellular reaction to lethal injury. In Methods and Achievements in Experimental Pathology, edited by E. Bajusz and G. Jasmin, Karger, Basel, New York, 4, pp. 1-29.

TRUMP/...

TRUMP, B.F., GOLDBLATT, P.J. and STOWELL, R.E. (1965). Studies on necrosis of mouse liver in vitro. Ultrastructural alterations in the mitochondria of hepatic parenchymal cells. Lab. Invest., 14, 343-371.

TUBIANA, M. and MALAISE, E.P. (1976). Growth rate and cell kinetics in human tumours: some prognostic and therapeutic implications. In Scientific Foundations of Oncology, edited by T. Symington and R.L. Carter, William Heinemann Medical Books Ltd., London, pp. 126-136.

TURNELL, R.W., CLARKE, L.H. and BURTON, A.F. (1973). Studies on the mechanism of corticosteroid-induced lymphocytolysis. Cancer Res., 33, 203-212.

WADDELL, A.W., BIRD, C.C. and CURRIE, A.R. (1976). Inhibition of uridine uptake by methylprednisolone in human lymphoblastoid cells. Biochem. Soc. Trans., 4, 262-264.

WADDELL, A.W., BIRD, C.C. and CURRIE, A.R. (1977a). The role of cytoplasmic glucocorticoid receptors in the cytolysis of human lymphoblastoid cells by methylprednisolone. Biochem. Soc. Trans., 5, 719-721.

WADDELL, A.W., BIRD, C.C. and CURRIE, A.R. (1977b). Effect of methylprednisolone on the nucleoside metabolism of a human lymphoblastoid cell line. Br. J. Cancer, 36, 187-191.

WADDELL, A.W., WYLLIE, A.H., ROBERTSON, A.M.G., MAYNE, K.M., AU, J. and CURRIE, A.R. (1979). Cytotoxic actions of glucocorticoids. In Proceedings of the VIIth Tenovus Workshop on Glucocorticoid Action and Leukaemia, edited by Bell and Borthwick, Alpha, Omega Press, Cardiff, in press.

WEINTRAUB/...

WEINTRAUB, L.R. (1972). Do remissions in acute leukemia depend upon "cell kill"? Arch. Intern. Med., 129, 498-499.

WERTHAMER, S. and AMARAL, L. (1975). Effect of cortisol on the ultrastructure of normal, leukemic, and cultured human lymphocytes. In Vitro, 11, 212-223.

WHITFIELD, J.F., PERRIS, A.D. and YOUDALE, T. (1968). Destruction of the nuclear morphology of thymic lymphocytes by the corticosteroid cortisol. Exp. Cell. Res., 52, 349-362.

WOLFF, J.A., BRUBAKER, C.A., MURPHY, M.L., PIERCE, M.I. and SEVERO, N. (1967). Prednisone therapy of acute childhood leukemia: prognosis and duration of response in 330 treated patients. J. Pediatr., 70, 626-631.

WYLLIE, A.H. (1974). Death in normal and neoplastic cells. J. Clin. Pathol., 27, Suppl. (Roy. Coll. Path.), 7, 35-42.

WYLLIE, A.H., KERR, J.F.R. and CURRIE, A.R. (1973). Cell death in the normal neonatal rat adrenal cortex. J. Pathol., 111, 255-261.

WYLLIE, A.H., KERR, J.F.R., MACASKILL, I.A.M. and CURRIE, A.R. (1973). Adrenocortical cell deletion: the role of ACTH. J. Pathol., 111, 85-94.

YAMAMOTO, K.R., STAMPFER, M.R. and TOMKINS, G.M. (1974). Receptors from glucocorticoid-sensitive lymphoma cells and two classes of insensitive clones: physical and DNA-binding properties. Proc. Natl. Acad. Sci., U.S.A. 71, 3901-3905.

YARBRO, G.S.K., LIPPMAN, M.E., JOHNSON, G.E. and LEVENTHAL, B.G. (1977). Glucocorticoid receptors in subpopulations of childhood acute lymphocytic leukemia. Cancer Res., 37, 2688-2695.

# APPENDIX I

## CALCULATION OF GROWTH KINETIC PARAMETERS (a) DOUBLING TIME AND (b) CUMULATIVE TOTAL CELL NUMBER, FOR MEASUREMENT OF GROWTH INHIBITORY RESPONSE OF HUMAN LYMPHOID CELLS TO METHYLPREDNISOLONE

(a) Doubling time ($t_{DT}$)

$$t_{DT} = \frac{\log 2}{K}$$ .....where K = growth constant

$$K = \frac{1}{t} \cdot \log \frac{N}{N_0}$$ .....where t = time interval

N = total number of cells at end of t

$N_0$ = total number of cells at beginning of t

but t = 48hr (interval between 'feedings' and cell counts)

$$\therefore t_{DT}\ (hr) = 48 . \frac{\log 2}{\log \frac{N}{N_0}}$$

(b) Cumulative total cell number

$$[N]_t = \frac{[N]_{t-48}}{n} \times N_t$$ .....where N = total cell number at end of 48hr interval

[N] = cumulative total cell number at end of 48hr interval

n = total cell concentration at beginning of 48hr interval

t = time (hr) = 48, 96, 144 ..... etc.

## APPENDIX II

### EFFECT OF A 30 MINUTE PULSE-LABEL WITH VARIOUS CONCENTRATIONS OF $^3$H-TdR ON GROWTH RATE OF $EB_4$ CELLS

This experiment was carried out under my supervision by Karen M. Mayne as part of her Honours Thesis (1977) entitled 'A study of the glucocorticoid-induced cytostatic response of a human lymphoblastoid cell line ($EB_4$)'. Most of the 'Materials and Methods' and 'Results' are already detailed in the text (Part II, Section 3), however more information is presented here.

Materials and Methods (see Part II, Section 3, p. 113)

The method of feeding cultures differed from that used for my own growth inhibitory experiments (Part II, Section 1, p. 82); cells were fed every 48hr by replacing 2/5 of 'old' medium with fresh growth medium but without diluting the cell concentration such that cells tended to approach plateau growth phase after only 5-6 days in culture. However, this period was long enough to assess an effect on the growth rate.

Results (see Part II, Section 3, p. 117)

Fig. 45 (p. 118) illustrates the effect of various concentrations of $^3$H-TdR on the growth rate of $EB_4$ cells over a period of 6 days. The smallest difference in growth rate between control and $^3$H-TdR-treated cells which is statistically significant was calculated by K. Mayne to be 8%. From fig. 45, therefore, the following table was compiled.

TABLE 14/...

TABLE 14 TIME INTERVAL DURING WHICH PULSE OF VARIOUS DOSES OF $^{3}$H-TdR HAS NO SIGNIFICANT EFFECT ON $EB_4$ CELL GROWTH RATE

| $^{3}$H-TdR concentration ($\mu$Ci/ml) | Length of time (hr) |
|---|---|
| 0.1 | 102 |
| 0.5 | 90 |
| 1.0 | 60 |
| 2.0 | 47 |
| 5.0 | 31 |

The data represent the mean of duplicate experiments.

0.1 $\mu$Ci/ml $^{3}$H-TdR is the only concentration which shows no significant drop in growth rate over 96hr, the time period in which the PLM experiment is performed.

## APPENDIX III

## CONVENTIONAL AUTORADIOGRAPHIC TECHNIQUES FOR MEASUREMENT OF THE DURATION OF THE CELL CYCLE AND ITS COMPONENT PHASES, AND THE GROWTH FRACTION

List of abbreviations:

| | | |
|---|---|---|
| $t_C$ | = | cell cycle time |
| $t_{G1}$ | = | duration of $G_1$ phase |
| $t_S$ | = | duration of S phase |
| $t_{G2}$ | = | duration of $G_2$ phase |
| $t_M$ | = | duration of mitosis (M) |
| $t_{C(pot)}$ | = | potential doubling time of population |
| $f_G$ | = | growth fraction |
| $t_{DT}$ | = | the overall population doubling time |
| MI | = | mitotic index |
| LI | = | labelling index |
| $^3$H-TdR | = | $^3$H-thymidine |
| ø | = | cell loss factor |

(a) The percentage of labelled mitoses (PLM) technique - pulse-labelling with $^3$H-TdR (Quastler and Sherman, 1959; Baserga and Wiebel, 1969; Mitchison, 1971; Aherne, Camplejohn and Wright, 1977; Steel, 1977)

Cells are pulse-labelled with $^3$H-TdR thereby labelling all cells which are in the S-phase at the time. The progress of the cohort of labelled cells is then followed round the cell cycle by assessing the PLM on autoradiographs taken in serial samples.

Theoretical/...

Theoretical curves obtained from the scoring of labelled mitoses, and hence the method by which the $t_C$ and its various phases are calculated, are shown in fig. 63. The labelled cohort of S-phase cells initially passes through $G_2$ during which time no labelled mitoses are present. It then passes through M where the proportion of labelled mitoses increases to 100% over a period equal to $t_M$. It remains at this level over a period equal to $t_S$ and then drops again to zero level over $t_M$ as all the labelled cells move out of M into $G_1$. As cells move through $G_1 \longrightarrow$ S and into the next cycle, the same pattern is repeated and $t_C$ is calculated as the time between the mid-points of the two peaks. The values of $t_{G2}$ and $t_S$ are calculated from the median 50% level, and $t_{G1}$ is then calculated from the equation:

$$t_{G1} + \tfrac{1}{2}t_M = t_C - (t_S + t_{G2} + \tfrac{1}{2}t_M).$$

In practice, there is variation in the slopes of the curves (measuring the rate of entry into the phases) and damping of the curves due to variation in the $t_C$ and its phases (see fig. 63).

The value of $t_M$ can be calculated from:

$$t_M = \frac{MI}{LI} \times t_S$$ (Denekamp, 1970)....where a measurement of MI and LI is taken from the first sampling point.

(b) $^3$H-TdR continuous-labelling technique (Baserga and Wiebel, 1969; Aherne, Camplejohn and Wright, 1977; Steel, 1977)

Theoretical curves obtained for the continuous-LI of proliferating cell populations are shown in fig. 64. At the time of addition of $^3$H-TdR, the initial LI represents the initial cohort of cells in S-phase i.e., the equivalent of a pulse-LI. The labelled population then/...

Fig. 63

Theoretical curves for percentage of labelled mitosis technique after pulse-labelling a population of proliferating cells with $^3$H-thymidine ($^3$H-TdR)

The percentage of labelled mitoses (PLM) is counted in successive samples. ▬▬, idealised curve where no variation exists among the cells between the cell cycle time and its individual phases; ——, more realistic curve, where variation in the cell cycle time and its phases causes variation in the slope of the curve and damping of the curve due to desynchronisation of the labelled cohort.
For further explanation see text p. 184.

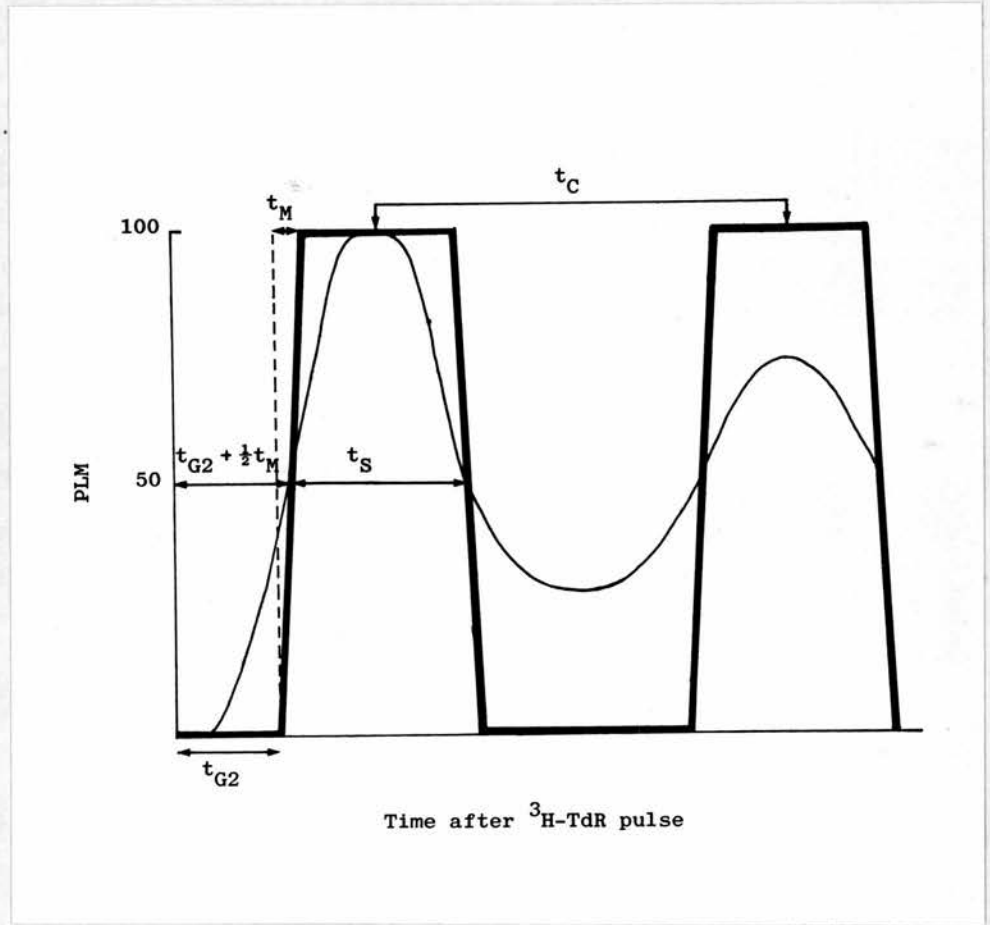
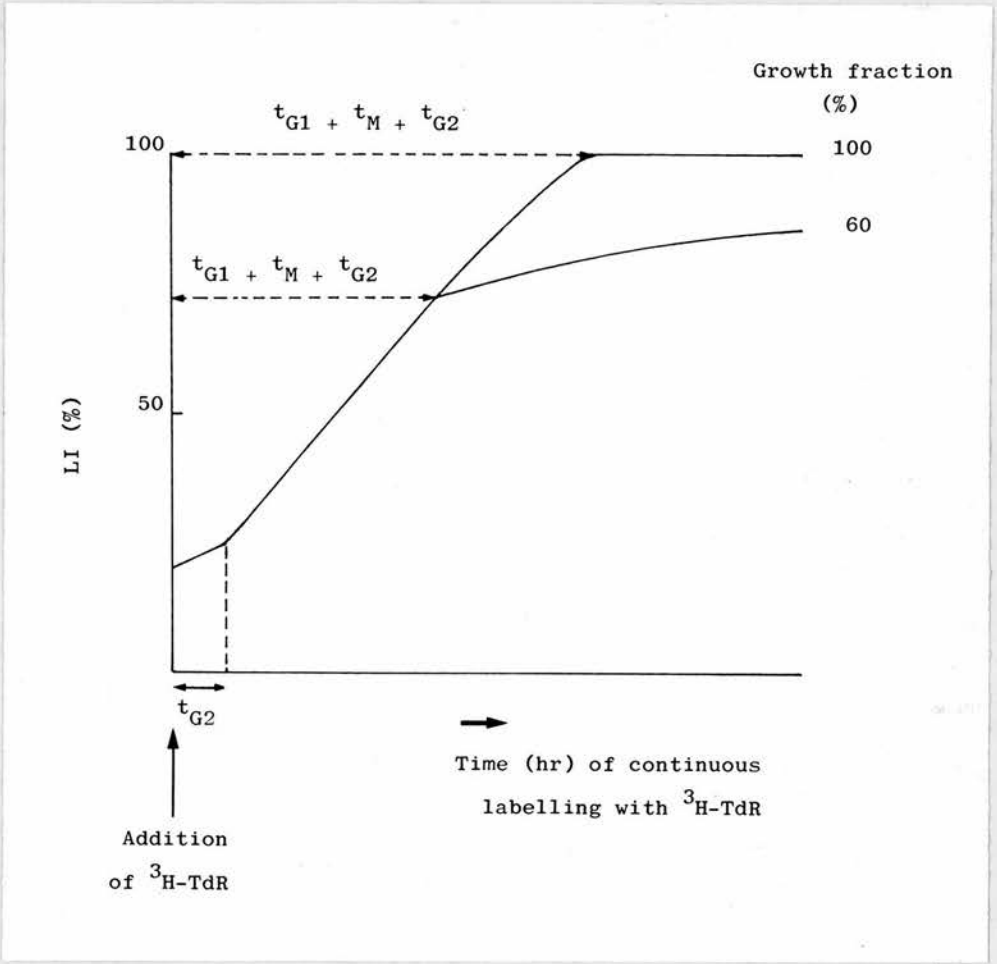


Fig. 64

Theoretical curves for the $^{3}$H-thymidine ($^{3}$H-TdR) continuous-labelling index (LI) of proliferating populations of cells

Adapted from Steel (1977, fig. 2.8, p. 77) where growth fraction and $t_{G1}$ vary between curves.

For further explanation see text, p. 185.



then proceeds through $G_2$ into mitosis where they divide and so cause the sudden observed increase in rate of appearance of labelled cells. As the population of $G_1$, M and $G_2$ cells proceed through to S then these too become labelled due to the continuous presence of $^3$H-TdR.

If the entire population is proliferating or if cell loss only occurs from the oldest non-proliferating cells then 100% labelling will be achieved after time $t_{G1} + t_{G2} + t_M$ as shown in fig. 64. However if $f_G < 100\%$, then the curve will asymptotically approach 100% labelling: when $G_1$ and $G_2$ cells enter S and become labelled, the only cells remaining unlabelled are non-proliferating cells which were present when labelled cells began to come through mitosis and which will progressively be diluted by growth of the dividing population. $f_G$ can therefore be found by extrapolating the more slowly rising component of the curve back to the end of $G_2$ (Steel, 1977) (fig. 64).

The technique of continuous-labelling can also be used to measure $t_{G2} + t_M$ which is the time needed to label all mitotic cells. This is estimated by assessing the PLM in successive samples and fig. 65 shows theoretical curves obtained from such data (this follows the same logic as for the initial part of the PLM curve after pulse-labelling with $^3$H-TdR (fig. 63).

$t_{G1}$ can then be calculated from these two sets of continuous-labelling data where:

$$t_{G1} = (t_{G1} + t_M + t_{G2}) - (t_{G2} + \tfrac{1}{2}t_M + \tfrac{1}{2}t_M)$$

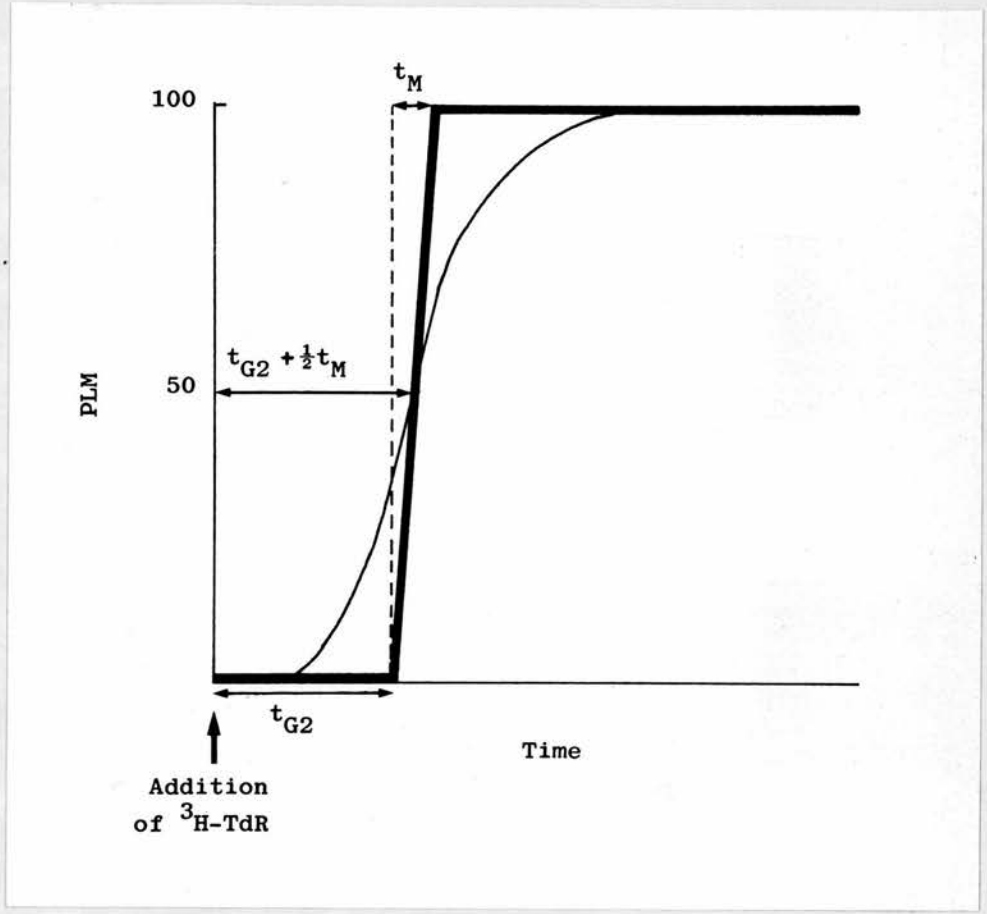
(c)/...

Fig. 65

Theoretical curves for the estimation of $t_{G2} \pm \frac{1}{2}t_M$ by continuous-labelling of a population of proliferating cells with $^3$H-thymidine ($^3$H-TdR)

The percentage of labelled mitoses (PLM) is counted in successive samples. ▬▬, idealised curve with no variation in $t_{G2}$; ——, more realistic curve where variation exists in the $t_{G2}$ values.

For further explanation see text, p. 188.



(c) Calculation of $f_G$, $t_{C(pot)}$ and $\emptyset$ from PLM - pulse-labelling curve

Two methods can be used to calculated these parameters:

(i) Mendelsohn, 1962

$$f_G = \frac{LI}{<PLM>}$$

....where LI can be calculated from the first autoradiograph sample

$<PLM>$ = mean or equilibrium value of PLM after flash-labelling with $^3$H-TdR

$<PLM>$ can be measured from the level on the curve at which the wave of PLM damps out to give a steady value. Alternatively, if the experiment is not continued long enough for sufficient damping to occur, then $<PLM>$ can be measured by averaging the values of PLM obtained over 1 cell cycle duration.

The equation above is derived from:

$$f_G = \frac{N_c}{N}$$

....where $N_c$ = number of cells cycling

$N$ = total number of cells

or rewritten,

$$f_G = \left(\frac{N_s}{N}\right) \Big/ \left(\frac{N_s}{N_c}\right)$$

....where $N_s$ = number of cells in S

$\frac{N_s}{N}$ = LI

$\frac{N_s}{N_c}$ = $<PLM>$, since when the labelled cohort of cells is desynchronised, the proportion of cells labelled in each of the cell cycle phases and in the cell cycle as a whole will be the same.

This/...

This method involves certain difficulties as:

(a) $<$PLM$>$ could be underestimated - in order to achieve complete desynchronisation, cells must go through several cell cycles during which time the number of grains/nucleus is halved such that a 'true' labelled cell has fewer grains than the estimated background grain count.

(b) $<$ PLM $>$ could be overestimated - if cells die, the label is released and taken up by other S-phase cells not in the original pulse-labelled cohort.

The $f_G$ can then be used to calculate $t_{C(pot)}$:

where $f_G = 1$, then $t_{C(pot)} = t_C$ (obtained from PLM curve)

where $f_G = <1$, then $t_{C(pot)} = \frac{t_C}{f_G}$

(ii) Steel, 1968

$$\ln (1 + f_G) = \frac{t_C}{t_{C(pot)}} \times \ln 2 \quad \text{....equation (i)}$$

where $t_{C(pot)} = \frac{\lambda . t_S}{LI}$ ....equation (ii)

$\lambda$ = correction factor to allow for the non-uniform age distribution of cells through the cell cycle in an exponentially growing population

$$= \frac{t_{C(pot)}}{t_S} \left[ e^{\left( \frac{\ln 2 \, (t_{G2} + t_S)}{t_{C(pot)}} \right)} - e^{\left( \frac{\ln 2 \, t_{G2}}{t_{C(pot)}} \right)} \right]$$

....equation (iii)

$\lambda$ is obtained by a series of successive approximations between equations (ii) and (iii).

Again, where $f_G < 1$, then $t_{C(pot)} > t_C$

Loss/...

Loss of cells by death can also occur in cultures. In populations with no cell loss, then

$$t_{C(pot)} = t_{DT}$$

However if cell loss occurs, then

$$t_{C(pot)} < t_{DT}$$

and the cell loss factor ($\phi$) is defined as

$$\phi = 1 - \frac{t_{C(pot)}}{t_{DT}}$$

References:

AHERNE, W.A., CAMPLEJOHN, R.S. and WRIGHT, N.A. (1977). In An Introduction to Cell Population Kinetics, Edward Arnold (Publishers) Ltd., London, pp. 27-43.

BASERGA, R. and WIEBEL, F. (1969). The cell cycle of mammalian cells. Int. Rev. Exp. Pathol., 7, 1-30.

DENEKAMP, J. (1970). The cellular proliferation kinetics of animal tumors. Cancer Res., 30, 393-400.

MENDELSOHN, M.L. (1962). Autoradiographic analysis of cell proliferation in spontaneous breast cancer of C3H mouse. III. The growth fraction. J. Natl. Cancer Inst., 28, 1015-1029.

MITCHISON, J.M. (1971). In The Biology of the Cell Cycle, Cambridge University Press, pp. 21-22.

QUASTLER, H. and SHERMAN, F.G. (1959). Cell population kinetics in the intestinal epithelium of the mouse. Exp. Cell Res., 17, 420-438.

STEEL, G.G. (1968). Cell loss from experimental tumours. Cell Tissue Kinet., 1, 193-207.

STEEL, G.G. (1977). In Growth Kinetics of Tumours, Clarendon Press, Oxford, pp. 76-79 and 120-145.

## PUBLICATIONS

The following work connected with this thesis has been published or is at present in preparation for publication -

BIRD, C.C., ROBERTSON, A.M.G., READ, J. and CURRIE, A.R. (1977). Cytolethal effects of glucocorticoids in human lymphoblastoid cell lines. J. Pathol., 123, 145 - 156.

BIRD, C.C., WADDELL, A.W., ROBERTSON, A.M.G., CURRIE, A.R., STEEL, C.M. and EVANS, J. (1975). Cytoplasmic receptor levels and glucocorticoid response in human lymphoblastoid cell lines. Br. J. Cancer, 32, 700 - 707.

ROBERTSON, A.M.G., BIRD, C.C., WADDELL, A.W. and CURRIE, A.R. (1978). Morphological aspects of glucocorticoid-induced cell death in human lymphoblastoid cells. J. Pathol., 126, 181 - 187.

WADDELL, A.W., NICHOLSON, H.R., DURIE, D.J.B., ROBERTSON, A.M.G. and WYLLIE, A.H. Intracellular ATP concentrations during glucocorticoid-induced lymphoid cell death. In preparation.

WADDELL, A.W., WYLLIE, A.H., ROBERTSON, A.M.G., MAYNE, K.M., AU, J. and CURRIE, A.R. (1979). Cytotoxic actions of glucocorticoids. In Proceedings of the VIIth Tenovus Workshop on Glucocorticoid Action and Leukaemia, edited by Bell and Borthwick, Alpha, Omega Press, Cardiff, in press.

*Br. J. Cancer* (1975) 32, 700

# CYTOPLASMIC RECEPTOR LEVELS AND GLUCOCORTICOID RESPONSE IN HUMAN LYMPHOBLASTOID CELL LINES

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**Summary.—The cytolethal response to treatment with prednisolone was investigated *in vitro* in eight human lymphoblastoid cell lines containing varying concentrations of specific cytoplasmic glucocorticoid receptors. A similar response was observed in seven of the lines irrespective of their concentration of cytoplasmic receptors, and pharmacological doses of steroid, well above those required to saturate receptors in cell-free extracts, were required for a massive lethal response. One cell line derived from Burkitt's lymphoma was refractory to lethal effects even with pharmacological doses of steroid.**

**A similar unresponsiveness to the cytolethal effect of prednisolone *in vitro* was observed in fresh lymphoblasts derived from patients with acute lymphoblastic leukaemia despite evidence of a satisfactory clinical response to therapy which included steroid. The resistance of human lymphoblastoid cells to treatment with glucocorticoids *in vitro* may result from a defect in activation subsequent to the binding of steroid to cytoplasmic receptors.**

THE cytolethal effects of glucocorticoid hormones on normal and neoplastic lymphoid cells are well established (Dougherty, 1952; Harris, 1970; Rosenau *et al.*, 1972). Moreover, in combination with other drugs, glucocorticoid hormones are highly effective in the treatment of acute lymphoblastic leukaemia (ALL) of man (Simone, 1974). At the molecular level, however, the precise mode of action of glucocorticoid hormones on lymphoid cells has still to be resolved. It is generally held that binding of steroid to specific protein receptor molecules in the cytoplasm is the first step in the cytolytic process in sensitive cells. Subsequently, steroid-receptor complexes are believed to undergo a temperature-dependent conformational change and migrate to the nucleus, where they influence transcriptional activity in such a way that cell lysis results (Munck *et al.*, 1972; Higgins *et al.*, 1973; Thompson and Lippman, 1974).

However, much of the current state of knowledge concerning the mechanism of glucocorticoid hormone action is based on experiments with rodent tissues, including thymocytes and various cultured cell lines. Little is known of these events in human lymphoid cells and, in particular, the role of cytoplasmic receptors in the initiation of hormone effects appears uncertain. In one study (Lippman *et al.*, 1973) with freshly isolated lymphoblasts from patients with ALL, a close correlation was found between hormone responsiveness *in vivo* and the concentration of cytoplasmic receptors. However, other studies (Gailani *et al.*, 1973; Lippman, Perry and Thompson, 1974) with 3 lymphoblastoid cell lines *in vitro*, failed to reveal such an association and the role of cytoplasmic receptors in the initiation of cytolethal effects by glucocorticoids in human cells remains to be established.

To investigate this problem, we have

studied the relationship of cytoplasmic receptor levels and glucocorticoid cytolethal effects in a series of human lymphoblastoid cell lines derived from patients with leukaemia or lymphoma, or without malignant disease.

## MATERIALS AND METHODS

*Cell lines.*—The cell lines were derived from freshly isolated human lymphoid cells of lymph glands, lymphoid tumours or peripheral blood. They were established as permanent cell lines in suspension culture, either spontaneously or by a process of co-cultivation with lethally irradiated cells containing Epstein–Barr virus (EBV) as described previously (Pulvertaft, 1965; Jensen *et al.*, 1967; Steel and Edmond, 1971; Steel, 1972). Previous studies have shown these cells to have the characteristics of B lymphocytes by their ability to synthesize immunoglobulins (Evans, Steel and Arthur, 1974), to have $C^{1}3$ receptors on their surface membranes (Moore and Minowada, 1973), to lack receptors for sheep red blood cells (Evans, Smith and Steel, 1975) and to be devoid of cytotoxic activity (Steel *et al.*, 1974).

*Cell culture.*—Cells were grown in suspension in conical glass flasks or roller culture bottles in Eagle's minimum essential medium (MEM, Gibco Biocult), or Ham's F10 medium with 10% tryptose phosphate broth (Gibco Biocult), supplemented with 20% heat-inactivated (56°C for 1 h) foetal calf serum (FCS, Gibco Biocult), at 37°C in a humidified atmosphere of 5% $CO_2$ in air. They were maintained at densities between 3 and $10 \times 10^5$/ml by feeding with fresh medium every 3–4 days.

*Chromosome analysis.*—Approximately $2 \times 10^6$ viable cells were resuspended in 5 ml of fresh growth medium. After 24 h, a drop of 0·02% dimethylcolchicine was added to the culture and the incubation continued at 37°C for a further 60–90 min. The cells were harvested by centrifugation, exposed to 0·0075 mol/l KCl for 10 min and fixed in 3 changes of methanol : glacial acetic acid (3 : 1, v/v). Drops of the fixed suspension were allowed to dry on clean slides, stained for 8 min in 0·5% quinacrine dihydrochloride, washed for 5 min in running water, mounted in distilled water under a sealed coverslip and examined with a Leitz Ortholux microscope with Ploem's vertical illumination using an HBO 200 u.v. source. Most cell lines have been examined repeatedly at intervals of a few months, and from 6 to 30 metaphase spreads photographed and fully analysed on each occasion.

*Steroid binding by cell extracts.*—The binding of glucocorticoid hormones to specific high affinity cytoplasmic receptors was studied by the competitive binding assay developed by Baxter and Tomkins (1971) using radioactively labelled and unlabelled dexamethasone. $3–5 \times 10^8$ cells were harvested by centrifugation (800 *g* for 10 min), washed twice in phosphate buffered saline (PBS; 0·025 mol/l $KH_2PO_4$, 0·1 mol l NaCl, pH 7·4) at 0–4°C, recentrifuged and homogenized in ice-cold tricine buffer (0·02 mol/l tricine, 0·002 mol/l $CaCl_2$, 0·001 mol l $MgCl_2$, pH 7·4). Rat thymuses were excised aseptically, rinsed in ice-cold PBS, blotted dry and chopped finely with scissors in 1 vol of ice-cold tricine buffer and homogenized. The cell and thymic homogenates were centrifuged at 105,000 *g* at 4°C for 1 h and duplicate aliquots of cytosol (0·4 ml) incubated at 0°C with varying concentrations of [1, 2(n)-$^3$H]-dexamethasone (19–29 Ci/mmol; Radiochemical Centre, Amersham) in the presence or absence of a 1000-fold excess of non-radioactive dexamethasone (Sigma). Unbound steroid was removed after 2 h by addition of 50–100 $\mu$l activated charcoal (200 mg/ml; BDH Chemicals), which was vigorously agitated for 5 sec and centrifuged (600 *g* for 1 min). The supernatant was recentrifuged (10,000 *g* for 5 min) and aliquots (200 $\mu$l) of supernatant assayed for radioactivity in a toluene-based scintillant containing Triton X-100 (33% v/v; Intertechnique) and butyl-PBD (5 g/l; Intertechnique) in a Beckman LS-250 liquid scintillation spectrometer (efficiency ~30%). Specifically bound dexamethasone represents the difference in amount of $^3$H-dexamethasone bound to cytosol in the absence and presence of 1000-fold excess of non-radioactive steroid. Protein concentration was measured by the technique of Lowry *et al.* (1951) using bovine serum albumin as standard.

*Cytolethal tests.*—Duplicate cultures of cells ($3–5 \times 10^5$/ml) were grown in MEM supplemented with 20% heat-inactivated FCS at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After 48 h when cells were in log phase of growth, methyl predni-

solone sodium succinate (Solumedrone, Upjohn) was added in aqueous solution at concentrations between $10^{-7}$ and $10^{-3}$ mol/l (final volume 1%). After incubation for a further 48 h. the total number of cells was enumerated with a haemacytometer and the viability assessed by exclusion of nigrosine (0·25%). Per cent lysis was calculated by comparison with control cultures which received no steroid.

*RNA synthesis.*—The effect of prednisolone on the incorporation of (5-$^3$H)-uridine ($^3$HU: 27 Ci/mmol; Radiochemical Centre, Amersham) into the acid-insoluble fraction of cells was estimated. Duplicate cultures of cells (3–5 × $10^5$/ml) were grown as described above. Solumedrone was added at concentrations between $10^{-6}$ and $10^{-3}$ mol/l to duplicate 1·0 ml aliquots of cells and after 1 h these were pulsed with 1·0 μCi/ml $^3$HU for 20 min. The cells were collected in microfibre glass filters in a sampling manifold (Millipore), precipitated with ice-cold 5% trichloroacetic acid (3 × 10 ml) and washed with ice-cold 70% ethanol (3 × 10 ml). Filters were dried at 37°C and assayed for radioactivity in a toluene-based scintillant containing butyl-PBD (5·0 g/l) in a Beckman LS-250 liquid scintillation spectrometer (efficiency ~ 30%). Results are expressed as incorporation of $^3$HU into the acid insoluble fraction/$10^6$ viable cells.

## RESULTS

### *Origin and karyotype of lymphoblastoid cell lines*

The origin, karyotype and age *in vitro* of the 8 cell lines used in our studies are shown in Table I. Whereas there was some variation in chromosome constitution within each line, there was always a clear modal karyotype. Four lines—$RUS_1$, $RUS_2$, $PEN_2$ and $YAK_1$—had only minor alterations to the normal diploid human complement, but the others had multiple breakages and recombinations, including fragments and abnormal chromosomes the precise origin of which could not be established.

### *Glucocorticoid cytoplasmic receptors in lymphoblastoid cell lines*

In steroid binding studies specific receptors in the cytoplasmic extracts (cytosol) of lymphoblastoid cells became saturated with dexamethasone at concentrations above 5–8 × $10^{-8}$ mol/l as illustrated in Fig. 1. Scatchard (1949) analysis of the data, shown in the insert of Fig. 1, yields a straight line consistent with a single class of receptor molecules

TABLE I.—*Origin, Karyotype, Age in Culture and Cytoplasmic Receptor Concentration of Human Lymphoblastoid Cell Lines*

| Cell line | Origin | Modal karyotype | Age in culture (mth) | Specifically bound dexamethasone (pmol/mg protein) |
|---|---|---|---|---|
| $RUS_1$ | Acute myeloblastic leukaemia | 46 XY 18p+ | 27 | 0·82 |
| $RUS_2$ | Acute myeloblastic leukaemia | 46 XY 3/8 Translocation | 27 | 0·66 |
| $BLA_1$ | Acute lymphoblastic leukaemia | 46 XY Multiple breakages and recombinations | 34 | 0·62 |
| F89 | Subacute lymphatic leukaemia | 48 XY Multiple breakages and recombinations | 94 | 0·16 |
| $GS_1$ | Chronic lymphatic leukaemia | 48 XX Multiple breakages and recombinations | 73 | 0·71 |
| J1JOYE | Burkitt's lymphoma | Near tetraploid. Multiple breakages and recombinations | 96 | 0·43 |
| $PEN_2$ | Adult blood* | 48 XXY 14+ | 24 | 0·09 |
| $YAK_1$ | Cord blood | 47 XY Partial trisomy 4 | 17 | 0·37 |
| Fresh thymus | Rat+ | | — | 0·33 |

The results shown are the mean of 2 separate determinations.
* Klinefelter's syndrome. + Female PVG/C rats aged 88 days.

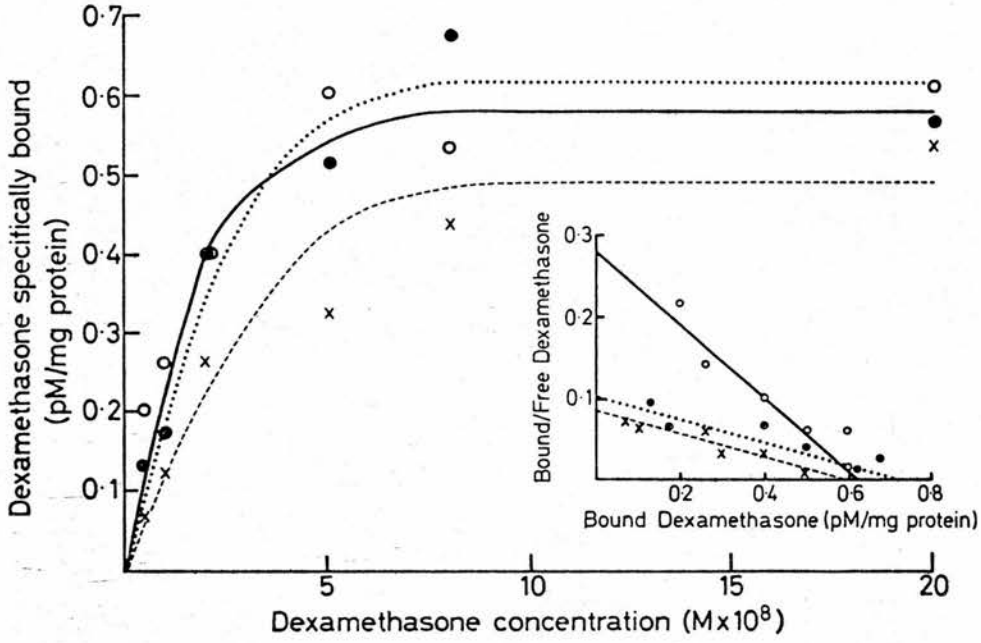


FIG. 1.—Specific binding of dexamethasone to cell-free extracts of human lymphoblastoid cell lines and rat thymus. Each point represents the mean of 3 separate experiments. The insert shows Scatchard plots of the data. ○———○, $RUS_2$ cells; ○- - - -○, $GS_1$ cells; ×-·-·-×, rat thymus.

of uniform steroid affinity. The equilibrium (dissociation) constants for the 2 examples shown, calculated from the intercepts of the reciprocal plots, were $1{\cdot}0 \times 10^{-8}$ mol/l ($RUS_2$) and $2{\cdot}3 \times 10^{-8}$ mol/l ($GS_1$). For comparison, Fig. 1 shows also the binding of dexamethasone to cytoplasmic receptors of fresh rat thymus, a tissue of known high sensitivity to the cytolytic effects of glucocorticoid hormones *in vivo* (Dougherty and White, 1945); saturation occurred at similar concentrations of steroid, and the dissociation constant ($3{\cdot}7 \times 10^{-8}$ mol/l) was of similar magnitude.

Further characterization of lymphoblastoid cell receptors revealed that they were thermolabile and completely inactivated by 30 min pre-incubation at 37°C. Similarly, incubation for 10 min at 20°C with trypsin (1 mg/ml) and protease (1 mg/ml) destroyed the binding capacity of cytosol. Incubation with deoxyribonuclease (bovine pancreas, 100 μg/ml) and ribonuclease (bovine pancreas, 100 μg/ml) had no significant effect on the binding characteristics. Thus, the cytoplasmic glucocorticoid receptors of human lymphoblastoid cells appear to be of a protein nature similar to those described in other glucocorticoid sensitive tissues (Hackney *et al.*, 1970; Munck and Wira, 1971; Baxter and Tomkins, 1971).

Using the competitive binding assay at saturating concentrations of dexamethasone ($8 \times 10^{-8}$ mol/l), the relative concentration of receptors in the cytosols of the various cell lines was determined. As shown in Table I a gradation in receptor concentration was found. The highest levels (0·62–0·82 pmol/mg protein) occurred in cell lines derived from patients with acute leukaemia and from one case of chronic lymphatic leukaemia, whilst intermediate concentrations (0·37–0·43 pmol/mg protein) were found in lines derived from a Burkitt's lymphoma and a healthy placental cord blood. The lowest levels (0·09–0·16 pmol/mg protein)

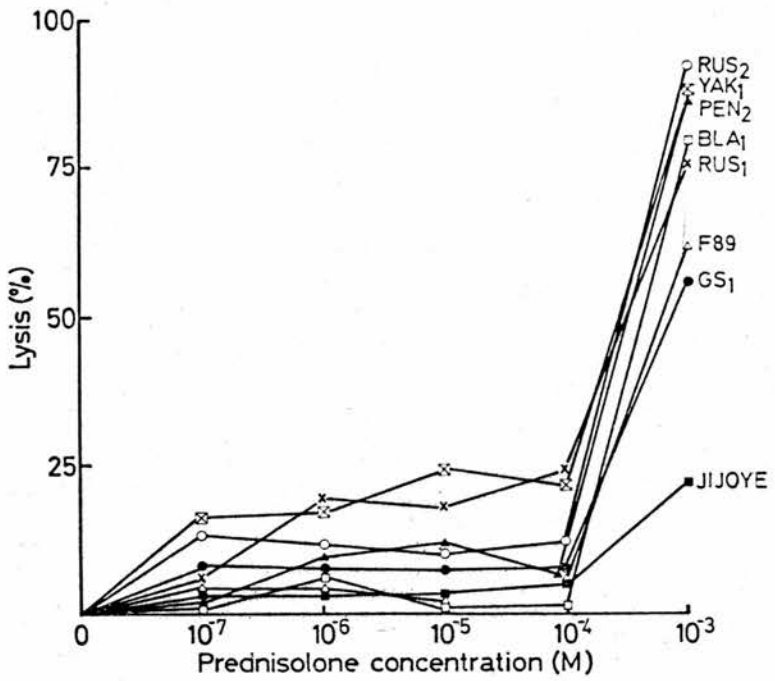


FIG. 2.—Cytolytic effect of prednisolone on human lymphoblastoid cell lines. Per cent lysis was calculated by comparison with control cultures which received no steroid. Each point represents the mean of 2 separate experiments.

were found in the lines derived from a patient with subacute lymphatic leukaemia and from the peripheral blood of a non-leukaemic adult patient. The concentration of receptors (0·33 pmol/mg protein) in the fresh rat thymus corresponded to the intermediate values obtained in the cell lines.

*Glucocorticoid cytolethal response*

The lethal response was assessed morphologically by the ability of cells to exclude the dye nigrosine, following incubation with aqueous preparations of steroid for 48 h. As shown in Fig. 2, a mild lethal response (10–15% of cells) was observed with prednisolone at concentrations of $10^{-7}$–$10^{-4}$ mol/l although these effects were apparently not in direct proportion to absolute concentrations of steroid. A marked increase in the cytolethal effect was observed, however, when the steroid concentration was increased to $10^{-3}$ mol/l and in some instances more than 85% of cells were killed. The magnitude of this enhanced lethal response, however, did not correlate with the measured levels of specific cytoplasmic hormone receptors, and some of the cell lines with low receptor concentration appeared to be as sensitive as those with high receptor levels (compare Fig. 2 and Table I). The cell line derived from Burkitt's lymphoma, however, was notably resistant to lethal effects even with high doses of steroid. Table I and Fig. 2 show also that no correlation could be established between steroid receptor levels or sensitivity to cytolytic effects and criteria which may be related to the malignant " potential " of lymphoblastoid cells *in vivo*, namely the origin of the cells (from malignant or non-malignant conditions), the degree of abnormality of modal karyotype or the age of cells *in vitro*.

The concentration of prednisolone

($10^{-3}$ mol l) required to achieve severe lethal effects exceeds physiological plasma levels of steroid ($10^{-6}$–$10^{-7}$ mol/l) by several orders of magnitude. Moreover, as can be seen in Fig. 1, it is considerably in excess of steroid concentrations required to saturate receptors in cytoplasmic extracts. However, when other glucocorticoid hormones such as cortisol and dexamethasone were tested over the same concentration range virtually the same, or in some cases somewhat reduced, lethal effects were obtained, and no significant differences were observed when steroids soluble in ethanol or dimethylsulphoxide were substituted for aqueous preparations. Furthermore, destruction of transcortin binding activity of serum with heat (56°C for 1 h) did not reduce the lethal response obtained with cortisol or prednisolone.

Ultrastructural studies of cultures treated with $10^{-3}$ mol/l prednisolone showed that less than 3% of steroid-treated cells contained EBV particles and the cytolethal effects could not be attributed to induction of virus lytic cycle.

Cytolethal tests were also performed with lymphoblasts isolated from the peripheral blood of 6 patients with ALL before commencement of therapy. Despite an apparent satisfactory clinical response to chemotherapy which included prednisolone, these cells did not show any greater sensitivity to the lethal effects of glucocorticoids *in vitro* than the cultured lymphoblasts. Insufficient material was available, however, to estimate the receptor levels in these cells.

*Glucocorticoid effect on RNA synthesis*

The effect of prednisolone on the incorporation of $^3$HU into the cold acid-insoluble fraction of lymphoblastoid cells was studied as an earlier and more sensitive index of cell damage than nigrosine. Preliminary investigations showed that significant inhibition of $^3$HU incorporation could be detected within 1 h of addition of prednisolone. Similar results were observed in all the cell lines studied, including the Burkitt's lymphoma cell line, as shown in Table II, irrespective of their specific cytoplasmic receptor concentration: thus, 1 h after addition of $10^{-5}$ and $10^{-6}$ mol/l steroid there was a slight reduction ($\leqslant$20%) in $^3$HU incorporation; with $10^{-4}$ mol/l prednisolone moderate reductions (30–50%) were observed whilst addition of $10^{-3}$ mol/l steroid produced a marked inhibition ($>$75%) of $^3$HU incorporation in all cell lines.

TABLE II.—*Effect of Prednisolone on Incorporation of $^3$H-uridine into Human Lymphoblastoid Cell Lines*

| Cell line | Control incorporation (ct/min/$10^6$ viable cells) | Fractional incorporation of control: Prednisolone concentration (mol/l) $10^{-6}$ | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ |
|---|---|---|---|---|---|
| $RUS_1$ | 2975 | 0·99 | 0·89 | 0·72 | 0·23 |
| $RUS_2$ | 7254 | 0·90 | 0·81 | 0·56 | 0·15 |
| $BLA_1$ | 13152 | 0·87 | 0·79 | 0·47 | 0·09 |
| F89 | 17743 | 1·02 | 0·86 | 0·58 | 0·13 |
| $GS_1$ | 5539 | 0·87 | 0·84 | 0·63 | 0·19 |
| J1JOYE | 45620 | 0·88 | 0·83 | 0·68 | 0·16 |
| $PEN_2$ | 12630 | 0·89 | 0·88 | 0·60 | 0·11 |
| $YAK_1$ | 15571 | 0·91 | 0·86 | 0·64 | 0·15 |

The results shown are the mean of two separate determinations and represent incorporation of $^3$H-uridine into the acid-insoluble fraction/$10^6$ viable cells.

## DISCUSSION

In contrast to the findings *in vivo* with lymphoblastoid cells of ALL patients (Lippman *et al.*, 1973), our results clearly show that the level of specific cytoplasmic receptors in human lymphoblastoid cells cannot be used to predict their responsiveness to glucocorticoid treatment *in vitro*. Similar responses to steroid treatment were obtained with all but one of the cell lines despite widely varying levels of cytoplasmic receptors: the exception was a cell line derived from Burkitt's lymphoma, although it showed a similar response to inhibition of RNA synthesis as the other cell lines. It is noteworthy that in our studies significant lethal effects were observed only with doses of steroid which produced a severe reduction ($>$75%) in incorporation of RNA

precursors. Other workers (Rosen *et al.*, 1972; Stevens, Stevens and Hollander, 1974) have claimed that smaller reductions in RNA synthesis are associated with impending lethal effects, although their experiments did not include morphological observations of cell death.

Although failure to exclude nigrosine is a rather insensitive test of cytolethal damage since it occurs late in the process of cell death, other techniques which employ release of specific radiolabels from damaged cells measure similar late phenomena and are associated with inherent interpretative difficulties due to "spontaneous" release of label ($^{51}$chromium) or internal radiation effects ($^{125}$ iododeoxyuridine).

When compared with rodent lymphoma cell lines, human lymphoblastoid cells appear relatively insensitive to the lethal effects of glucocorticoids *in vitro*. Rodent lymphoma cell lines (Harris, 1970; Rosenau *et al.*, 1972; Turnell, Clarke and Burton, 1973; Kondo, Kikuta and Noumura, 1975), nearly always show marked lethal responses to concentrations of glucocorticoids in the physiological range ($10^{-6}$–$10^{-7}$ mol/l) and thus may differ fundamentally in their biological responsiveness to steroid hormones.

The failure to correlate cytoplasmic receptor levels with glucocorticoid responses, and the requirement of pharmacological doses of steroid for substantial cytolethal effects, suggest that cytoplasmic receptors may not be responsible for initiation of the lethal glucocorticoid effects we have observed in human lymphoblastoid cells. Alternatively, some form of steroid resistance may have developed during the long period of cultivation of cells *in vitro*. However, in our hands freshly isolated lymphoblasts from ALL patients showed a similar resistance to lethal glucocorticoid effects *in vitro*. It is possible, therefore, that defects in activation of glucocorticoid cytolethal methanisms may occur in lymphoblastoid cells cultured *in vitro* for short or long periods of time, rendering cells insensitive to all but massive doses of steroid.

Until recently, the emergence of resistance to steroid effects has been attributed to quantitative reductions in cytoplasmic receptor levels (Rosenau *et al.*, 1972; Lippman *et al.*, 1973). Clearly, in our cell lines this cannot account for steroid resistance if present. However, Sibley and Tomkins (1974) have recently shown in studies with steroid-resistant clones of mouse lymphoma cells that whilst resistance to steroid effects results predominantly from quantitative deficiencies in steroid receptors, other more subtle defects in hormone activation may occur. Thus, resistance may result from qualitative defects in cytoplasmic receptor molecules or reduction in the capacity for transfer of formed steroid receptor complexes to the nucleus. Rarely, defects in the specific localization of complexes within the nucleus appear to occur since nuclear binding of steroid receptor complexes did not provoke a lethal response in some clones.

It is evident, therefore, that the binding of steroids to cytoplasmic receptors represents only one stage of a complex series of events leading to expression of hormone effects. It remains to be seen whether the activation of steroids in human lymphoblastoid cells *in vitro* differs fundamentally from that occurring *in vivo*. It seems likely, however, that analysis of each step in the activation process will be required before the potential responsiveness of cells to glucocorticoid hormones can be predicted accurately.

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## REFERENCES

BAXTER, J. D. & TOMKINS, G. M. (1971) Specific Cytoplasmic Glucocorticoid Hormone Receptors in Hepatoma Tissue Culture Cells. *Proc. natn. Acad. Sci. U.S.A.*, **68**, 932.

DOUGHERTY, T. F. (1952) Effect of Hormones on Lymphatic Tissue. *Physiol. Rev.*, **32**, 379.

DOUGHERTY, T. F. & WHITE, A. (1945) Functional Alterations in Lymphoid Tissue Induced by Adrenal Cortical Secretion. *Am. J. Anat.*, **77**, 81.

EVANS, J., SMITH, M. A. & STEEL, C. M. (1975) Glutaraldehyde and Human T Cell Rosettes. *J. Immunol. Methods.* In the press.

EVANS, J., STEEL, M. & ARTHUR, E. (1974) A Haemagglutination Inhibition Technique for Detection of Immunoglobulins in Supernatants of Human Lymphoblastoid Cell Lines. *Cell*, **3**, 153.

GAILANI, S., MINOWADA, J., SILVERNAIL, P., NUSSSBAUM, A., KAISER, N., ROSEN, F. & SHIMAOKA, K. (1973) Specific Glucocorticoid Binding in Human Hemopoietic Cell Lines and Neoplastic Tissue. *Cancer Res.*, **33**, 2653.

HACKNEY, J. F., GROSS, S. R., ARONOW, L. & PRATT, W. B. (1970) Specific Glucocorticoid-binding Macromolecules from Mouse Fibroblasts Growing *In vitro*. A Possible Steroid Receptor for Growth Inhibition. *Molec. Pharmacol.*, **6**, 500.

HARRIS, A. W. (1970) Differentiated Functions Expressed by Cultured Mouse Lymphoma Cells. I. Specificity and Kinetics of Cell Responses to Corticosteroids. *Expl cell Res.*, **60**, 341.

HIGGINS, S. J., ROUSSEAU, G. G., BAXTER, J. D. & TOMKINS, G. M. (1973) Early Events in Glucocorticoid Action. Activation of the Steroid Receptor and its Subsequent Specific Nuclear Binding Studied in a Cell-free System. *J. biol. Chem.*, **248**, 5866.

JENSEN, E. M., KOROL, W., DITTMAR, S. L. & MEDREK, T. J. (1967) Virus Containing Lymphocyte Cultures from Cancer Patients. *J. natn. Cancer Inst.*, **39**, 745.

KONDO, H., KIKUTA, A. & NOUMURA, T. (1975) Studies on Glucocorticoid-induced Cytolysis in Cultured Mouse Lymphoma Cells, L5178Y. I. Glucocorticoid Specificity and Cytoplasmic Receptors in Sensitive and Resistant Cells. *Expl cell Res.*, **90**, 285.

LIPPMAN, M. E., HALTERMAN, R. H., LEVENTHAL, B. G., PERRY, S. & THOMPSON, E. B. (1973) Glucocorticoid-binding Proteins in Human Acute Lymphoblastic Leukemic Blast Cells. *J. clin. Invest.*, **52**, 1715.

LIPPMAN, M. E., PERRY, S. & THOMPSON, E. B. (1974) Cytoplasmic Glucocorticoid-binding Proteins in Glucocorticoid-unresponsive Human and Mouse Leukemic Cell Lines. *Cancer Res.*, **34**, 1572.

LOWRY, O. H., ROSEBROUGH, N. H., FARR, A. L. & RANDALL, R. J. (1951) Protein Measurement with the Folin Phenol Reagent. *J. biol. Chem.*, **193**, 265.

MOORE, G. E. & MINOWADA, J. (1973) B and T Lymphoid Cell Lines. *New Engl. J. Med.*, **288**, 106.

MUNCK, A. & WIRA, C. (1971) Glucocorticoid Receptors in Rat Thymus Cells. *Adv. Biosci.*, **7**, 301.

MUNCK, A., WIRA, C., YOUNG, D. A., MOSHER, K. M., HOLLAHAN, C. & BELL, P. A. (1972) Glucocorticoid-receptor Complexes and the Earliest Steps in the Action of Glucocorticoids on Thymus Cells. *J. Ster. Biochem.*, **3**, 567.

PULVERTAFT, R. J. V. (1965) A Study of Malignant Tumours in Nigeria by Short-term Tissue Culture. *J. clin. Path.*, **18**, 261.

ROSEN, J. M., FINA, J. J., MILLHOLLAND, R. J. & ROSEN, F. (1972) Inhibitory Effect of Cortisol *In vitro* on 2-Deoxyglucose Uptake and RNA and Protein Metabolism in Lymphosarcoma P1798. *Cancer Res.*, **32**, 350.

ROSENAU, W., BAXTER, J. D., ROUSSEAU, G. G. & TOMKINS, G. M. (1972) Mechanism of Resistance to Steroids: Glucocorticoid Receptor Defect in Lymphoma Cells. *Nature, New Biol.*, **237**, 20.

SCATCHARD, G. (1949) The Attraction of Proteins for Small Molecules and Ions. *Ann. N.Y. Acad. Sci.*, **51**, 660.

SIBLEY, C. H. & TOMKINS, G. M. (1974) Mechanisms of Steroid Resistance. *Cell*, **2**, 221.

SIMONE, J. (1974) Acute Lymphocytic Leukemia in Childhood. *Semin. Hematol.*, **11**, 25.

STEEL, C. M. (1972) Human Lymphoblastoid Cell Lines. III. Co-cultivation Technique for Establishment of New Lines. *J. natn. Cancer Inst.*, **48**, 623.

STEEL, C. M. & EDMOND, E. (1971) Human Lymphoblastoid Cell Lines. I. Culture Methods and Examination for Epstein–Barr Virus. *J. natn. Cancer Inst.*, **47**, 1193.

STEEL, C. M., HARDY, D. A., LING, N. R. & LAUDER, I. J. (1974) The Interaction of Normal Lymphocytes and Cells from Lymphoid Cell Lines. VI. Line-directed Cytotoxic Specificity of Lymphocytes Activated by Autochthonous or Allogeneic LCL Cells. *Immunology*, **26**, 1013.

STEVENS, J., STEVENS, Y. W. & HOLLANDER, V. P. (1974) Substrate Requirements and Kinetic Analysis of the Cortisol Effects on Uridine Uptake and Incorporation by Mouse Lymphoma P1798 Cells *In vitro*. *Cancer Res.*, **34**, 2330.

THOMPSON, E. B. & LIPPMAN, M. E. (1974) Mechanism of Action of Glucocorticoids. *Metabolism*, **23**, 159.

TURNELL, R. W., CLARKE, L. H. & BURTON, A. F. (1973) Studies on the Mechanism of Corticosteroid-induced Lymphocytolysis. *Cancer Res.*, **33**, 203.

# CYTOLETHAL EFFECTS OF GLUCOCORTICOIDS IN HUMAN LYMPHOBLASTOID CELL LINES

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GLUCOCORTICOID hormones are employed extensively in the treatment of lymphoid neoplasms of man (DeVita, 1973; Simone, 1974; Schein *et al.*, 1975). It has been tacitly assumed that in human cells cytolethal hormone action is achieved through the same mechanisms as those which produce cytolysis of rodent lymphoid cells: after penetration of the plasma membrane steroid is thought to bind rapidly to specific cytoplasmic protein receptors to form complexes which transfer to the nucleus and initiate a sequence of molecular events culminating in a lethal response (Munck and Wira, 1971; Munck *et al.*, 1972; Higgins *et al.*, 1973; Thompson and Lippman, 1974). Certain steroid-resistant rodent lymphoma cells have quantitative deficiencies in cytoplasmic receptors whereas others have defects either in the nuclear transfer of complexes or in their activation within the nucleus (Kirkpatrick, Milholland and Rosen, 1971; Rosenau *et al.*, 1972; Sibley and Tomkins, 1974; Kondo, Kikuta and Noumura, 1975).

Specific cytoplasmic glucocorticoid receptors have been detected also in freshly isolated lymphoblasts from patients with acute lymphoblastic leukaemia and responsiveness *in vivo* to drug combinations, which included glucocorticoids, appeared to correlate with the concentration of cytoplasmic steroid receptors (Lippman *et al.*, 1973). However, studies with human lymphoblastoid cell lines (Gailani *et al.*, 1973; Lippman, Perry and Thompson, 1974; Bird *et al.*, 1975) have failed to confirm any direct association between cytoplasmic receptor levels and glucocorticoid responsiveness *in vitro*. Furthermore, to obtain lethal effects we had to use doses of glucocorticoids which greatly exceeded both physiological steroid levels and the binding capacity of receptors in cytosol extracts of cells.

We have, therefore, studied the optimum conditions for obtaining glucocorticoid-induced lethal effects in human lymphoblastoid cells *in vitro* and have also examined the kinetics of the response.

## MATERIALS AND METHODS

### *Cell lines*

Human lymphoblastoid cell lines were established in culture either spontaneously or by a process of co-cultivation with lethally irradiated cells containing Epstein-Barr virus as previously described (Pulvertaft, 1965; Jensen *et al.*, 1967; Steel and Edmond, 1971; Steel,

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1972). The cells were grown in suspension cultures in glass flasks or roller culture bottles in bicarbonate-buffered Eagle's minimum essential medium (MEM, GIBCO Bio-Cult, Scotland) supplemented with 20 per cent. heat-inactivated (56° for 1 hr) foetal calf serum (FCS, GIBCO Bio-Cult, Scotland) at 37° in a humidified atmosphere of 5 per cent. $CO_2$ in air. Stock cultures were maintained at densities between 3 and $10 \times 10^5$ cells/ml by feeding with fresh medium every 3–4 days.

### *Glucocorticoids*

The glucocorticoids used were: methylprednisolone sodium succinate (Solumedrone, Upjohn, England); dexamethasone sodium phosphate (Decadron, Merck, Sharp and Dohme, England); hydrocortisone sodium succinate (Solucortef, Upjohn, England); these were dissolved in sterile distilled water at the required concentrations. Prednisolone was a gift from Organon, Scotland, and was dissolved in dimethylsulfoxide (DMSO) or 95 per cent. ethanol. The cells were able to tolerate concentrations of up to 1 per cent. ethanol or DMSO without deleterious effects on their growth rate.

### *Steroid binding in cells*

The specific binding of steroid to whole cells was determined after the method of Sibley and Tomkins (1974). Cells in logarithmic growth phase were harvested by centrifugation at $800 \times g$ for 3 min. and aliquots containing $1 \times 10^6$ viable cells (assessed by the ability to exclude nigrosine) resuspended in tubes with 0·5 ml of medium (37°) containing 20 per cent. heat-inactivated FCS. (1, 2(n)-$^3$H)-dexamethasone (27–29 Ci/mM; Radiochemical Centre, Amersham, England) with or without a 1000-fold excess of non-radioactive dexamethasone (Sigma, England) was added in 95 per cent. ethanol (final concentration 1 per cent.) to give the desired steroid concentrations. The tubes were gassed with 5 per cent. $CO_2$ in air, stoppered and incubated at 37° in a shaking water bath at 60 revolutions/min. When the incubation was complete for the time specified, the cells were centrifuged ($800 \times g$ for 3 min.), washed immediately with 5 ml phosphate-buffered saline (PBS; 0·025M $KH_2PO_4$, 0·1M NaCl; *p*H 7·4) at 25° and recentrifuged ($800 \times g$ for 3 min.); thereafter they were washed three times with 5 ml of ice-cold PBS. The cell pellets were finally resuspended in ice-cold PBS and duplicate aliquots (100$\mu$l) assayed for radioactivity in 10 ml of toluene based scintillant containing Triton X-100 (33 per cent. v/v; Intertechnique, England) and butyl-PBD (5 g/l; Intertechnique, England) in a Beckman LS-250 liquid scintillation spectrometer (efficiency $\sim$ 30 per cent.). Specifically bound dexamethasone represents the difference in counts retained by $1 \times 10^6$ viable cells in the absence and presence of a 1000-fold excess of non-radioactive steroid.

### *Cytolethal tests*

In cytolethal tests duplicate cultures of cells in logarithmic growth phase were treated with various concentrations of glucocorticoids. After incubation for 48 hr, the total number of cells was enumerated with a haemocytometer or Coulter Counter and the viability was assessed by the ability of the cells to exclude nigrosine (0·25 per cent.).

### *Kinetics of lethal response*

Duplicate cultures of cells in logarithmic growth phase were treated with lethal concentrations of glucocorticoid for 48 hr. Samples were removed at selected time intervals throughout the experiment, and the total cell number and viability assessed as above. In the experiments where the duration of exposure to glucocorticoid was varied, cultures were washed three times (at 37°) with fresh medium after the desired period of exposure to steroid and the cell pellet resuspended in the original volume of fresh medium which contained no steroid. The cell viability was assessed before washing and at intervals afterwards up to 48 hr.

## RESULTS

### *Steroid binding in whole cells*

We have shown prevously (Bird *et al.*) that the cytoplasm of human lymphoblastoid cells contains protein receptors to which dexamethasone binds with high affinity and in a saturable fashion (table). Specific binding of dexamethasone in intact cells occurred in all cell lines with the same high affinity and at comparable levels of saturation ($1–2 \times 10^{-7}$M) as with cytoplasmic extracts of cells (fig. 1). Furthermore, uptake of steroid by cells at 37° was rapid, and maximal incorporation was reached within 5–10 min. exposure to a saturating concentration of steroid (fig. 2).

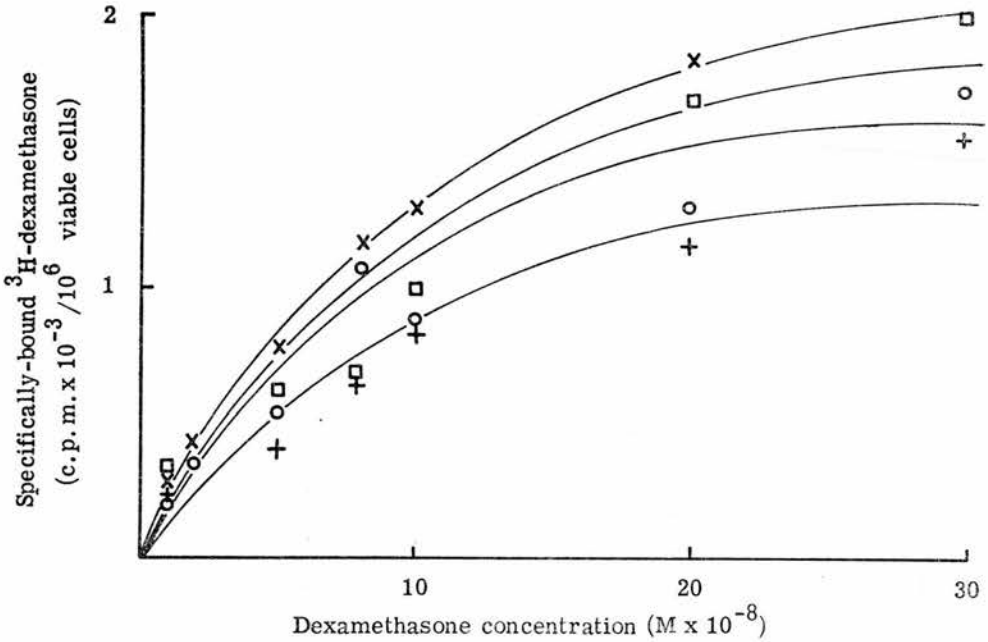


FIG. 1.—Specific binding of $^3$H-dexamethasone in whole lymphoblastoid cells. Samples of cells were incubated in growth medium at 37° in the presence of various concentrations of $^3$H-dexamethasone with or without a 1000-fold excess of non-radioactive dexamethasone. After 1 hr cells were washed with buffer and assayed for radioactivity. $BLA_1$ cells (○——○); $RUS_2$ cells (×——×); $GS_1$ cells (+——+); JIJOYE cells (□——□). Each point represents the mean of either two or three separate experiments.

### *Glucocorticoid sensitivity of cell lines*

To determine the relative sensitivity of cells to glucocorticoids, cultures were exposed to increasing concentrations of methylprednisolone sodium succinate for 48 hr. All cell lines showed a similar lethal response which was unrelated to the concentration of receptors measured previously in cytoplasmic extracts. Lethal effects were minimal (5–10 per cent. of cells) with steroid concentrations within the physiological and usual pharmacological range ($10^{-5}–10^{-7}$M); and

continued exposure of cells to steroid at these concentrations for up to 168 hr did not augment the lethal response. However, when the steroid was increased to $10^{-3}$M there was a massive lethal response (fig. 3) with $LD_{50}$ levels for cell lines ranging from $0{\cdot}71\times10^{-3}$M to $0{\cdot}99\times10^{-3}$M (table).

TABLE

*Origin, concentration of specific cytoplasmic steroid receptors and $LD_{50}$ values for human lymphoblastoid cell lines treated with methylprednisolone sodium succinate*

| Cell line | Origin | Concentration of cytoplasmic steroid receptors* (p M/mg protein) | $LD_{50}\times10^{-3}$M† |
|---|---|---|---|
| $GS_1$ | Chronic lymphatic leukaemia | 0·71 | 0·84±0·14 |
| $RUS_2$ | Acute myeloblastic leukaemia | 0·66 | 0·71±0·12 |
| $BLA_1$ | Acute lymphoblastic leukaemia | 0·62 | 0·79±0·03 |
| JIJOYE | Burkitt's lymphoma | 0·43 | 0·99±0·12 |

* From Bird *et al.*, 1975.
† The concentration of methylprednisolone at which lysis occurs in 50 per cent. of the cells tested. Results are the mean of three experiments ±SD.

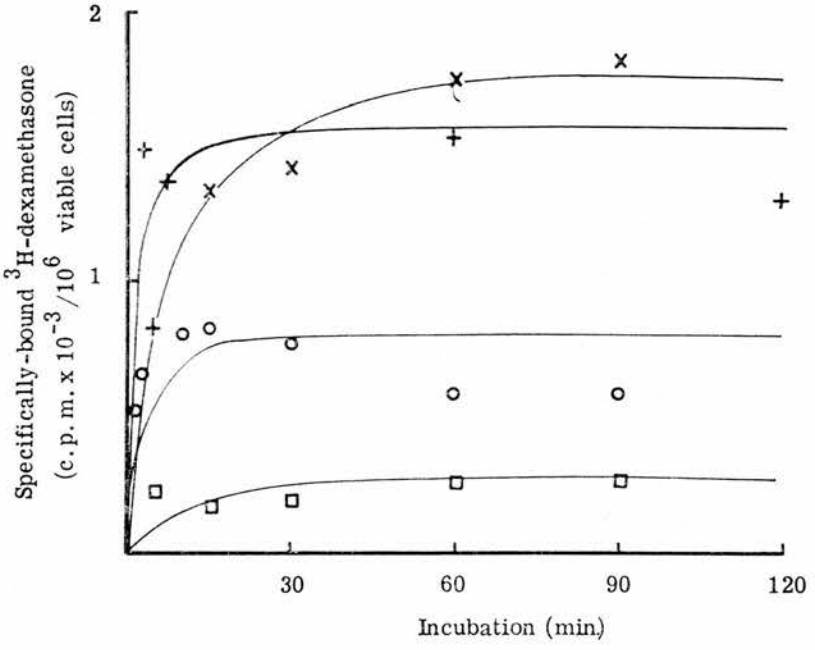


FIG. 2.—Specific binding of dexamethasone at various times following incubation of whole lymphoblastoid cells with $1\times10^{-7}$M $^3$H-dexamethasone with or without a 1000-fold excess of non-radioactive dexamethasone. Cells were washed with buffer at the times shown and assayed for radioactivity. $BLA_1$ (○——○); $RUS_2$ (×——×); $GS_1$ (+——+); JIJOYE (□——□). Each point represents the mean of three separate experiments.

The results illustrated in fig. 3 were obtained at cell concentrations within the optimal range for obtaining maximal lethal responses (*vide infra*).

### *Cell density and glucocorticoid sensitivity*

Cytolethal tests were performed with $10^{-3}$M methylprednisolone sodium succinate in cultures where the cell concentration was adjusted to $2–20 \times 10^5$

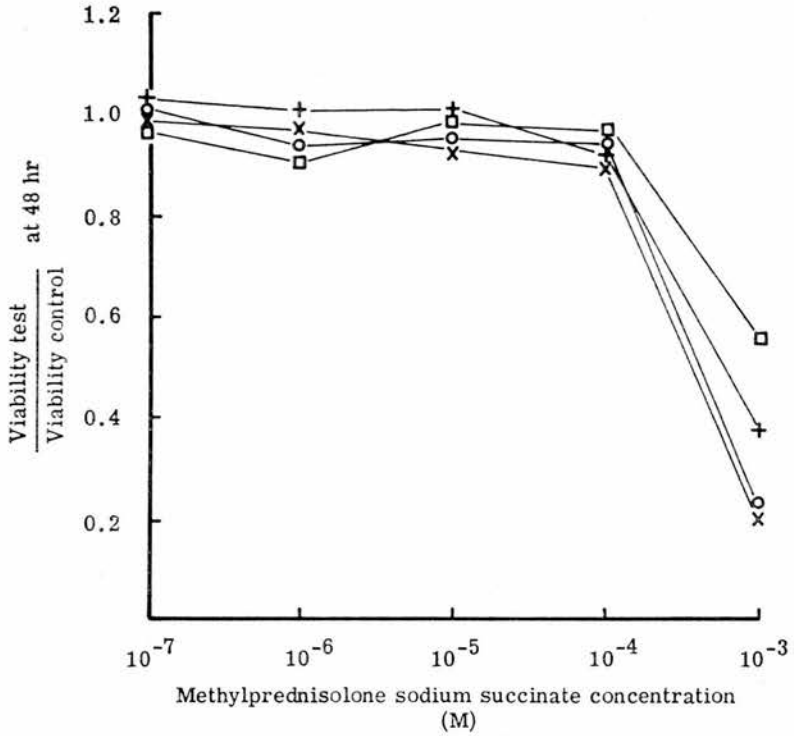


FIG. 3.—Lethal effects of methylprednisolone sodium succinate on human lymphoblastoid cell lines. Exponentially growing cells were treated with various concentrations of steroid for 48 hr when cell viability was assessed. $BLA_1$ (○——○); $RUS_2$ (×——×); $GS_1$ (+——+); JIJOYE (□——□). Each point represents the mean of three separate experiments.

cells/ml before addition of steroid. The sensitivity of cells to steroid was approximately in inverse proportion to the concentration of cells in cultures although differences were slight where cultures contained $2–10 \times 10^5$ cells/ml (fig. 4).

### *Steroid solvent and glucocorticoid sensitivity*

Within the concentration range of $10^{-6}–10^{-4}$M, preparations of prednisolone in ethanol or DMSO were no more effective than aqueous solutions of methylprednisolone sodium succinate in producing a lethal response (fig. 5). With

$10^{-3}$M, all preparations showed a massive lethal response, the most severe effects being achieved with methylprednisolone sodium succinate.

*Cytolethal response to different glucocorticoids*

Of the several glucocorticoid preparations tested, the most severe lethal effects were obtained in cultures treated with methylprednisolone sodium succinate; by comparison hydrocortisone sodium succinate was consistently

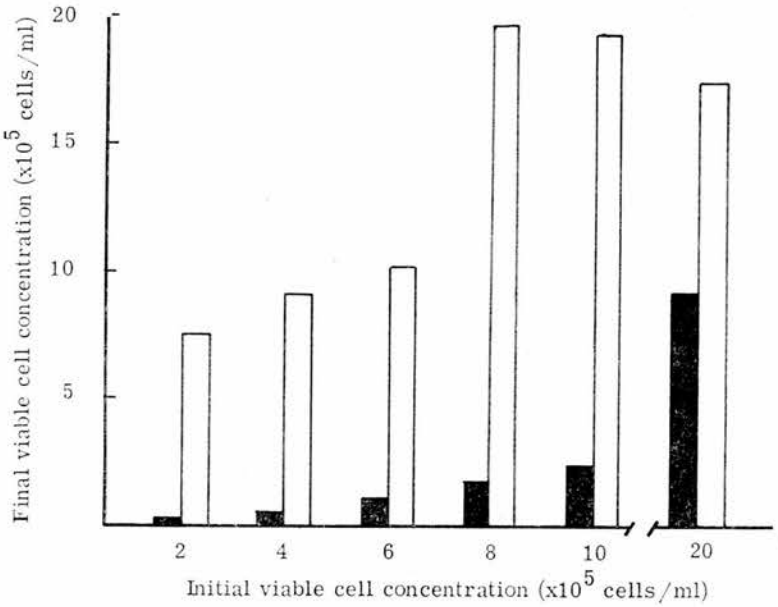


FIG. 4.—Effect of initial cell density of $RUS_2$ cells on lethal effect obtained with methylprednisolone sodium succinate ($10^{-3}$M); cells were incubated with steroid for 48 hr when cell viability and total cell concentration were assessed. Solid bar, methylprednisolone sodium succinate treated; open bar, control. Each bar represents the mean of three separate experiments.

much less potent than the other glucocorticoids (fig. 6). Inactivation of any residual specific glucocorticoid binding by globulins in the serum by heating for 1 hr at 56° before adding it to the culture medium had no significant influence on the cytolethal response obtained with either hydrocortisone sodium succinate or methylprednisolone sodium succinate.

*Kinetics of lethal glucocorticoid response*

We studied the kinetics of the lethal response to methylprednisolone sodium succinate at concentrations within the range $0{\cdot}75\times10^{-3}$–$2\times10^{-3}$M—known to produce a lethal effect by 48 hr. With the higher concentrations ($1\times10^{-3}$–$2\times10^{-3}$M) a brief lag phase was observed before progressive lethal effects ensued (fig. 7), whilst with the lower concentration ($0{\cdot}75\times10^{-3}$M) there was a more protracted latent interval.

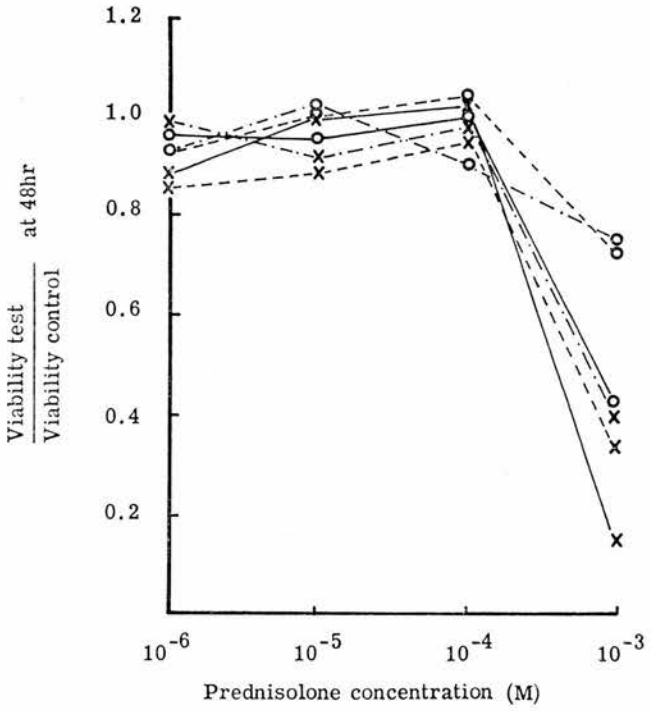


FIG. 5.—Lethal effect of substituted (water-soluble) and unsubstituted (ethanol- and dimethylsulfoxide - soluble) preparations of prednisolone on $BLA_1$ (○——○) and $RUS_2$ (×——×) cells. Exponentially growing cells were incubated with various concentrations of steroid dissolved in water (———), ethanol (– – – – –) or dimethylsulfoxide (–·–·–·–·–) for 48 hr when cell viability was assessed. Each point represents the mean of two separate experiments.

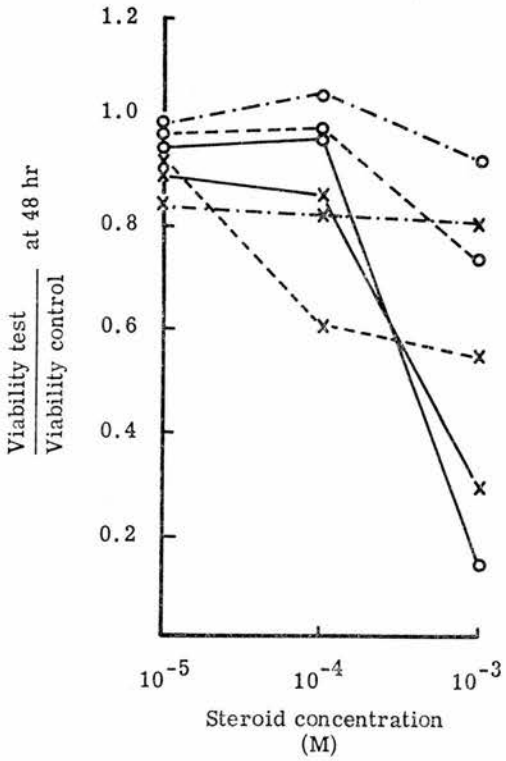


FIG. 6.—Lethal effects of various glucocorticoids on $BLA_1$ (○——○) and $RUS_2$ (×——×) cells. Exponentially growing cells were incubated with methylprednisolone sodium succinate (———), dexamethasone sodium phosphate (– – – – –) or hydrocortisone sodium succinate (–·–·–·–·–) for 48 hr when cell viability was assessed. Each point represents the mean of two separate experiments.

*Duration of glucocorticoid pulse and lethal response*

The duration of the glucocorticoid pulse to which cells were exposed was varied by washing cultures free of a lethal dose of methylprednisolone sodium succinate ($10^{-3}$M) at intervals up to 48 hr. Continued exposure to steroid was necessary for progression of lethal effects (fig. 8).

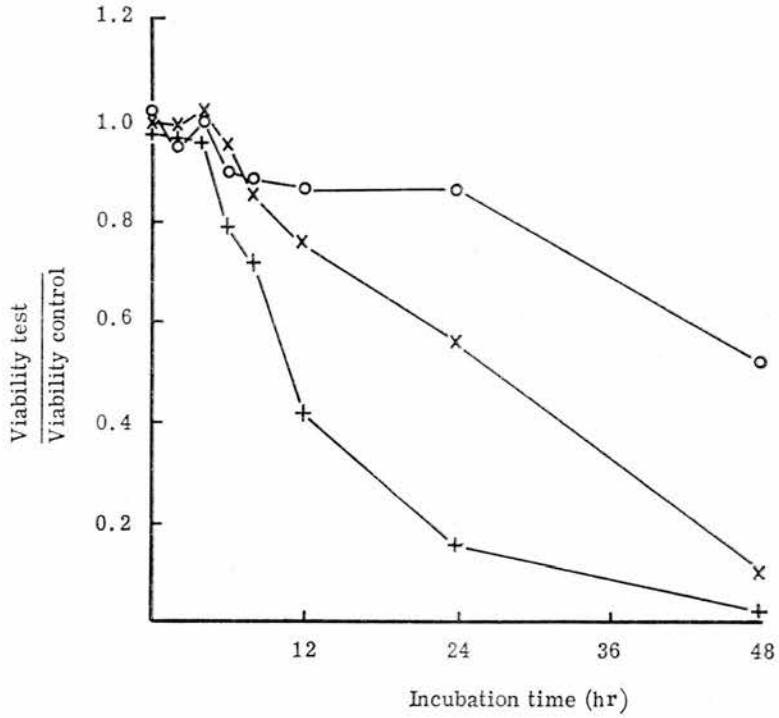


FIG. 7.—Kinetics of glucocorticoid lethal response in $BLA_1$ cells treated with $0{\cdot}75\times10^{-3}$M (○——○), $1\times10^{-3}$M (×——×) or $2\times10^{-3}$M (+——+) of methylprednisolone sodium succinate. Samples were removed at the times shown and assessed for viability. Each point represents duplicate observations from one typical experiment.

## DISCUSSION

*Steroid binding*

Under our culture conditions steroid penetrated readily into whole cells and was retained with the same high affinity and at comparable levels of saturation as previously demonstrated in cytoplasmic extracts of disrupted lymphoblastoid cells. However, the role of cytoplasmic receptors in initiating lethal steroid responses has still to be resolved. It seems clear that there is no direct correlation between the absolute levels of cytoplasmic receptors in cells and their sensitivity to lethal steroid effects. The complex nature of steroid activation has been revealed in recent studies with mouse lymphoma cells (Sibley and Tomkins, 1974) and it seems possible that resistance to lethal hormone effects can occur at several points in the hormone activation pathway. Recent

work has suggested that reductions in growth rate *in vitro* may be a more sensitive indicator of cell death *in vivo* than uptake of supravital dyes (Bhuyan *et al.*, 1976; Roper and Drewinko, 1976). We are currently investigating the relationship between nuclear binding of receptor-steroid complexes and cytostasis *in vitro*; preliminary results suggest that this may have a more direct bearing on cell deletion *in vivo*.

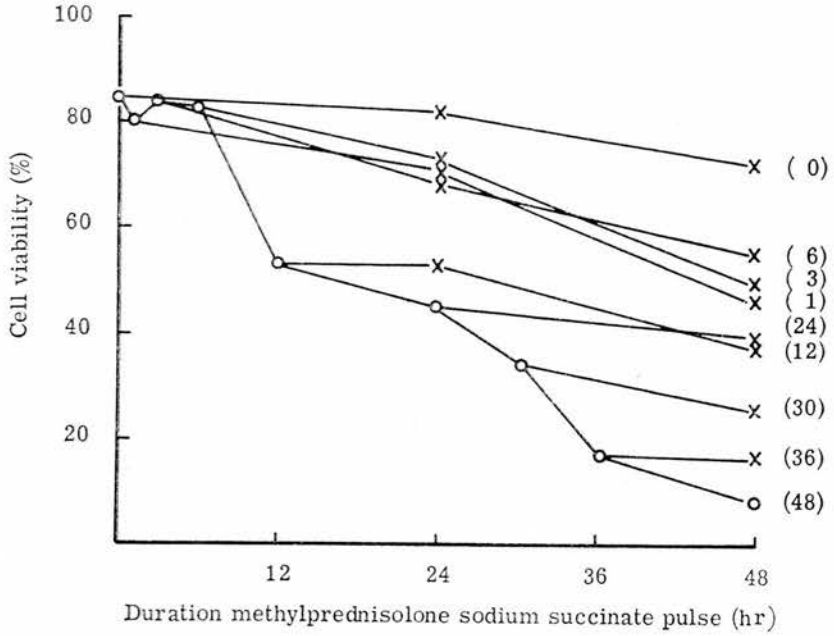


FIG. 8.—Duration of methylprednisolone sodium succinate ($10^{-3}$M) pulse and cytolethal effect on $RUS_2$ cells. Cells were washed free of steroid at the times shown (parenthesis). Cell viability was assessed before washing (○——○) and at intervals afterwards (×——×) up to 48 hr. Each point represents duplicate observations from one typical experiment.

We have also shown that inhibition of uridine and thymidine uptake into $BLA_1$ cells is virtually instantaneous with a lethal concentration of methylprednisolone sodium succinate ($1{\cdot}4\times10^{-3}$M) (Waddell, Bird and Currie, 1976, 1977). Methylprednisolone sodium succinate appears to act by competitively inhibiting facilitated diffusion uptake of uridine and thymidine. Since the extent of inhibition is independent of the concentration of cytoplasmic receptors, and is too rapid to allow time for alterations in transcription and translation, this also indicates that cytoplasmic receptors are not involved. The " cell surface " is a possible alternative target for the action of methylprednisolone sodium succinate in our system.

### *Glucocorticoid sensitivity of cell lines*

These studies confirm our previous observation (Bird *et al.*, 1975) that lethal effects in human lymphoblastoid cell lines and in fresh peripheral blood lymphoblasts from patients with acute lymphoblastic leukaemia can only be achieved

*in vitro* with doses of glucocorticoids which greatly exceed normal physiological and pharmacological levels. Assuming that equilibration of steroids within the fluid compartments of the body is equal and non-concentrative, the highest pharmacological doses of glucocorticoids employed in clinical practice (100–500 mg/m$^2$ body surface) would achieve peak intracellular concentrations around $1\text{–}5\times10^{-5}$M. Maximum physiological plasma steroid levels are generally considered to be about $10^{-6}$–$10^{-7}$M, much of which is probably bound to plasma corticosteroid binding globulin (transcortin) (Burton and Westphal, 1972). Thus human lymphoblastoid cells *in vitro* exhibit marked resistance to concentrations of glucocorticoids which are normally considered to have cytolethal activity *in vivo*. In this respect they differ fundamentally from rat thymocytes and mouse lymphoma cells which are much more sensitive to concentrations of steroid in the physiological and pharmacological range (Harris, 1970; Munck and Wira, 1971; Rosenau *et al.*, 1972; Turnell, Clarke and Burton, 1973).

Our studies also show that the insensitivity of cultured human lymphoblastoid cells to glucocorticoids cannot be attributed to the steroid preparation, steroid solvent or cell density at which routine cytolethal tests are performed. All experiments were performed at optimal cell concentrations with methylprednisolone sodium succinate in aqueous solution, the most potent of the glucocorticoid preparations examined; and extension of the period of exposure of cells to steroid for as long as 168 hr did not significantly augment the cytolethal response. This suggests that either lethal steroid mechanisms *in vitro* differ fundamentally from those pertaining *in vivo* or the pharmacological activity of glucocorticoids *in vivo* does not involve a direct cytolethal action.

### *Kinetics of lethal glucocorticoid response*

Kinetic studies of the lethal steroid hormone response *in vitro* reveal patterns suggestive of a dose-dependent relationship with maximal lethal effects occurring only where there is continuous exposure of cells to the steroid. Whilst the possibility remains that the resistance of human lymphoblastoid cell lines to lethal steroid effects results from a change in biological sensitivity induced by the long peroid of cultivation of cells *in vitro*, our previous studies have shown that fresh lymphoblasts from patients with acute lymphoblastic leukaemia exhibit similar resistance *in vitro* despite an apparent clinical response to drug combinations which included pharmacological doses of steroid (Bird *et al.*). It seems possible, therefore, that the pharmacological concentrations of steroid routinely employed in clinical practice may be insufficient to produce significant lethal effects *per se* but in combination with other chemotherapeutic agents act in a synergistic fashion to achieve a massive cytolethal response.

## SUMMARY

Glucocorticoid hormones penetrate rapidly into intact lymphoblastoid cells and are retained with the same high affinity and specificity as with cytoplasmic extracts.

Optimal conditions for lethal glucocorticoid responses *in vitro* were determined for a series of human lymphoblastoid cell lines by using different glucocorticoid preparations, and steroid solvents and by varying the cell density. Kinetic studies revealed that lethal glucocorticoid effects are dose-dependent and that continuous exposure of cells to steroid is necessary for progression of lethal effects to occur.

Even under optimal conditions, human lymphoblastoid cells only exhibit a marked cytolethal response to glucocorticoids at concentrations which greatly exceed both physiological and therapeutically attainable steroid levels. They are also greatly in excess of steroid levels required to saturate the cytoplasmic receptors of intact or disrupted lymphoblastoid cells. It is suggested that either lethal steroid mechanisms *in vitro* differ fundamentally from those *in vivo* or the pharmacological activity of glucocorticoids *in vivo* does not involve a direct cytolethal action.

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## REFERENCES

BIRD, C. C., WADDELL, A. W., ROBERTSON, A. M. G., CURRIE, A. R., STEEL, C. M., AND EVANS, J. 1975. Cytoplasmic receptor levels and glucocorticoid response in human lymphoblastoid cell lines. *Br. J. Cancer*, **32**, 700.

BHUYAN, B. K., LOUGHMAN, B. E., FRASER, T. J., AND DAY, K. J. 1976. Comparison of different methods of determining cell viability after exposure to cytotoxic compounds. *Expl. Cell Res.*, **97**, 275.

BURTON, R. M., AND WESTPHAL, U. 1972. Steroid hormone-binding proteins in blood plasma. *Metabolism*, **21**, 253.

DEVITA, V. T. 1973. Combined drug treatment of Hodgkin's disease: remission induction, remission duration, and survival; appraisal. *Natn. Cancer Inst. Monogr.*, **36**, 373.

GAILANI, S., MINOWADA, J., SILVERNAIL, P., NUSSBAUM, A., KAISER, N., ROSEN, F., AND SHIMAOKA, K. 1973. Specific glucocorticoid binding in human hemopoietic cell lines and neoplastic tissue. *Cancer Res.*, **33**, 2653.

HARRIS, A. W. 1970. Differentiated functions expressed by cultured mouse lymphoma cells. I. Specificity and kinetics of cell responses to corticosteroids. *Expl. Cell Res.*, **60**, 341.

HIGGINS, S. J., ROUSSEAU, G. G., BAXTER, J. D., AND TOMKINS, G. M. 1973. Early events in glucocorticoid action. Activation of the steroid receptor and its subsequent specific nuclear binding studied in a cell-free system. *J. Biol. Chem.*, **248**, 5866.

JENSEN, E. M., KOROL, W., DITTMAR, S. L., AND MEDREK, T. J. 1967. Virus containing lymphocyte cultures from cancer patients. *J. Natn. Cancer Inst.*, **39**, 745.

KIRKPATRICK, A. F., MILHOLLAND, R. J., AND ROSEN, F. 1971. Stereospecific glucocorticoid binding to subcellular fractions of the sensitive and resistant lymphosarcoma P1798. *Nature, New Biol.*, **232**, 216.

KONDO, H., KIKUTA, A., AND NOUMURA, T. 1975. Studies on glucocorticoid-induced cytolysis in cultured mouse lymphoma cells, L5178Y. I. Glucocorticoid specificity and cytoplasmic receptors in sensitive and resistant cells. *Expl. Cell Res.*, **90**, 285.

LIPPMAN, M. E., HALTERMAN, R. H., LEVENTHAL, B. G., PERRY, S., AND THOMPSON, E. B. 1973. Glucocorticoid-binding proteins in human acute lymphoblastic leukemic blast cells. *J. Clin. Invest.*, **52**, 1715.

LIPPMAN, M. E., PERRY, S., AND THOMPSON, E. B. 1974. Cytoplasmic glucocorticoid-binding proteins in glucocorticoid-unresponsive human and mouse leukemic cell lines. *Cancer Res.*, **34**, 1572.

MUNCK, A., AND WIRA, C. 1971. Glucocorticoid receptors in rat thymus cells. *Adv. Biosci.*, **7**, 301.

MUNCK, A., WIRA, C., YOUNG, D. A., MOSHER, K. M., HOLLAHAN, C., AND BELL, P. A. 1972. Glucocorticoid-receptor complexes and the earliest steps in the action of glucocorticoids on thymus cells. *J. Ster. Biochem.*, **3**, 567.

PULVERTAFT, R. J. V. 1965. A study of malignant tumours in Nigeria by short-term tissue culture. *J. Clin. Path.*, **18**, 261.

ROPER, P. R., AND DREWINKO, B. 1976. Comparison of *in vitro* methods to determine drug-induced cell lethality. *Cancer Res.*, **36**, 2182.

ROSENAU, W., BAXTER, J. D., ROUSSEAU, G. G., AND TOMKINS, G. M. 1972. Mechanism of resistance to steroids: glucocorticoid receptor defect in lymphoma cells. *Nature, New Biol.*, **237**, 20.

SCHEIN, P. S., CHABNER, B. A., CANELLOS, G. P., YOUNG, R. C., BERARD, C., AND DEVITA, V. T. 1975. Results of combination of chemotherapy of non-Hodgkin's lymphoma. *Br. J. Cancer*, **31**, Suppl. II, 465.

SIBLEY, C. H., AND TOMKINS, G. M. 1974. Mechanisms of steroid resistance. *Cell*, **2**, 221.

SIMONE, J. 1974. Acute lymphocytic leukemia in childhood. *Semin. Haematol.*, **11**, 25.

STEEL, C. M. 1972. Human lymphoblastoid cell lines. III. Co-cultivation technique for establishment of new lines. *J. Natn. Cancer Inst.*, **48**, 623.

STEEL, C. M., AND EDMOND, E. 1971. Human lymphoblastoid cell lines. I. Culture methods and examination for Epstein-Barr virus. *J. Natn. Cancer Inst.*, **47**, 1193.

THOMPSON, E. B., AND LIPPMAN, M. E. 1974. Mechanism of action of glucocorticoids. *Metabolism*, **23**, 159.

TURNELL, R. W., CLARKE, L. H., AND BURTON, A. F. 1973. Studies on the mechanism of corticosteroid-induced lymphocytolysis. *Cancer Res.*, **33**, 203.

WADDELL, A. W., BIRD, C. C., AND CURRIE, A. R. 1976. Inhibition of uridine uptake by methylprednisolone in human lymphoblastoid cells. *Biochem. Soc. Trans.*, **4**, 262.

WADDELL, A. W., BIRD, C. C., AND CURRIE, A. R. 1977. The role of cytoplasmic glucocorticoid receptors in the cytolysis of human lymphoblastoid cells by methylprednisolone. *Biochem. Soc. Trans.*, **5**, 719.

# MORPHOLOGICAL ASPECTS OF GLUCOCORTICOID-INDUCED CELL DEATH IN HUMAN LYMPHOBLASTOID CELLS

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PLATES LI–LV

IN both normal and neoplastic tissues cell death is an important parameter in growth kinetics (Steel, 1967; Laird, 1969) but little is known about the mechanisms involved.

*Coagulative necrosis* (McLean, McLean and Judah, 1965; Saladino and Trump, 1968; Hawkins *et al.*, 1972; McDowell, 1972) and in-vitro autolysis (Trump and Ginn, 1969) result from the breakdown of normal homeostatic control mechanisms, and usually occur with total disruption of structure of affected areas. *In vivo* there is usually associated inflammation in surrounding viable tissues (Majno, 1964).

*Apoptosis*, a morphologically distinctive type of cell death involved in cell deletion *in vivo* (Kerr, Wyllie and Currie, 1972), affects scattered single cells without surrounding structural damage or inflammation. In some situations it appears to play a role complementary to mitosis in the controlled regulation of normal (Wyllie *et al.*, 1973) and tumour cell populations (Kerr *et al.*).

It is well established that glucocorticoid hormones exert cytolethal effects on normal and neoplastic lymphoid cells (Dougherty and White, 1945; Burton, Storr and Dunn, 1967; Whitfield, Perris and Youdale, 1968), and these steroids are employed extensively in the treatment of lymphoid tumours in man (DeVita, 1973; Simone, 1974; Schein *et al.*, 1975). The mechanisms by which they induce tumour regression have yet to be resolved.

Since it is not possible to study sequential biochemical events in man *in vivo*, we have been investigating the biochemical and associated morphological changes in glucocorticoid-induced cell death of human lymphoblastoid cells *in vitro* (Bird *et al.*, 1975; Bird *et al.*, 1977; Waddell, Bird and Currie, 1977). This system involves cell death in a controlled environment and furthermore is uncomplicated by the presence and action of phagocytic cells.

We here record the morphological aspects of cell death induced by a cytolethal concentration of methylprednisolone sodium succinate (500 $\mu$g/ml) on a cell line ($BLA_1$) derived from a patient with acute lymphoblastic leukaemia.

## MATERIALS AND METHODS

*Cell culture.* The $BLA_1$ cell line was established from the peripheral blood of a patient with acute lymphoblastic leukaemia; its characteristics have been described previously

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(Bird *et al.*, 1975). The cells were grown in suspension culture in conical glass flasks or roller culture bottles in Eagle's minimum essential medium (MEM, GIBCO Bio-cult, Scotland) supplemented with 20 per cent. heat-inactivated (56°C for 1 hr) foetal calf serum (FCS, GIBCO Bio-cult, Scotland) at 37°C in a humidified atmosphere of 5 per cent. $CO_2$ in air. Stock cultures were maintained at densities between 2 and $10\times10^5$ cells/ml by feeding with fresh medium every 3–4 days.

*Glucocorticoid.* Methylprednisolone sodium succinate (MPS) (Solumedrone, Upjohn, England) was dissolved in sterile distilled water.

*Morphological studies.* Cultures of $BLA_1$ cells in logarithmic growth phase were treated with MPS to give a final concentration of 500 $\mu$g/ml steroid. Control cultures were treated with the appropriate volume of sterile distilled water.

*Cell preparation for light microscopy (LM).* Samples were removed sequentially over a 48-hr treatment period (see table). Smears of $2–5\times10^5$ cells were prepared on glass slides, air-dried, fixed in 95 per cent. methanol for 30 min., stained with 4 per cent. (v/v) Giemsa (Gurr's Improved R66, Searle Diagnostic, England) for 10 min. and mounted in Harleco synthetic resin (Harleco, Belgium).

*Cell preparation for transmission electron microscopy (TEM).* Samples were removed sequentially over a 48-hr treatment period as for light microscopy (see table). $50–100\times10^5$ cells were centrifuged at $400\times g$ for 5 min. at room temperature, and the resulting pellet resuspended in 1 ml of reconstituted sterile frozen-dried human plasma. All subsequent procedures, unless otherwise mentioned, were carried out at 4°C. Three per cent. gluteraldehyde (TAAB, England) in 0·2M sodium cacodylate buffer (BDH, England) at *p*H 7 was added, the suspension fixed for 1 hr and then centrifuged at $2000\times g$ for 5 min. to form a compact pellet. After further fixation for 2 hr, the pellet was washed in 0·2M sodium cacodylate buffer for 2 hr, post-fixed for 1 hr in 1 per cent. osmium tetroxide (Johnson Matthey, England) in 0·2M sodium cacodylate buffer, rewashed in buffer for 30 min. and dehydrated in increasing concentrations of ethanol. The cells were resuspended in epoxy-propane (BDH, England) at room temperature, and then impregnanted and set in epon resin (TAAB, England). Thin sections were cut on an LKB Ultrotome I with glass knives and mounted on formvar (TAAB, England) coated grids (Athene-type old 400). These were stained with saturated uranyl acetate (BDH, England) in 50 per cent. ethanol for 15 min. followed with 0·04M lead citrate (BDH, England) in 0·02M sodium hydroxide for 5 min. Sections were viewed on an AEI Corinth 275 electron microscope.

*Cell preparation for scanning electron microscopy (SEM).* Samples were removed at 0·5, 1, 2, 3, 4, 6 and 12 hr after treatment. The cells were centrifuged gently ($250\times g$ for 5 min.) and resuspended at $50\times10^5$ cells/ml in the medium in which they were grown. One drop of this suspension was placed at either end of a $6\times35$ mm coverslip and allowed to settle for 30 min. The cells were fixed by addition of one drop of 1 per cent. osmium tetroxide in 0·2M cacodylate buffer, and after 30 min. were gradually dehydrated by passing through increasing concentrations of acetone, and stored in 100 per cent. acetone. The samples were dried by critical point drying, which involved the substitution of acetone with liquid $CO_2$ and raising the temperature until it was above the critical point at which there is instantaneous evaporation of the $CO_2$. After coating with gold, the samples were viewed in a Cambridge Stereoscan 180 microscope.

## RESULTS

### *LM and TEM observations on lethal glucocorticoid response*

*Control cultures.* $BLA_1$ cells were round, oval or elongated with occasional cytoplasmic protrusions and long slender surface processes; cytoplasmic vacuoles were common (fig. 1). Ultrastructurally (fig. 2), the cytoplasm was rich in free polysomes and contained scanty profiles of rough endoplasmic reticulum, mitochondria with plate-like cristae, Golgi apparatus and lipid

droplets. Cells showed a high nuclear to cytoplasmic ratio and the nuclei, usually one per cell, were of variable shape and size with chromatin dispersed diffusely throughout the nucleoplasm and with one or more nucleoli (figs. 1 and 2). Scanty aggregates of heterochromatin were seen round the periphery of the nucleus (fig. 2). Small numbers of necrotic cells were also observed (*vide infra*).

*Treated cultures.* No significant differences were observed from control cultures until 1 hr after incubation with 500 $\mu$g/ml MPS (table) when two distinctive morphological changes were seen.

The first type of change, whose incidence was markedly increased from 1 to 6 hr after treatment with a peak at 2 hr (table), involved " blebbing " of the cells. The cells showed loss of long slender surface processes with contortion and blebbing of the cytoplasm and indentation of the nucleus, which sometimes showed pyknosis and fragmentation (fig. 3). The " blebbing " cells then broke

TABLE

*Percentage morphological types in smear preparations of $BLA_1$ cell cultures during treatment with 500 $\mu$g/ml methylprednisolone over 48 hr*

| Time after treatment (hr) | Percentage* morphological type | | | |
|---|---|---|---|---|
| | Normal | " Blebbing " | " Rounding-up " | Degenerate cells and degenerate fragments with nuclear remnants |
| 0 | 93·5 | 0·1 | 0·5 | 5·9 |
| 0·5 | 94·8 | 0·1 | 0·4 | 4·7 |
| 1 | 90·1 | 2·6 | 1·6 | 5·7 |
| 2 | 79·1 | 4·6 | 7·5 | 8·8 |
| 3 | 71·8 | 3·2 | 12·1 | 12·9 |
| 4 | 67·4 | 2·9 | 14·4 | 15·3 |
| 6 | 62·6 | 1·2 | 11·0 | 25·2 |
| 8 | 58·9 | 0·6 | 8·4 | 32·1 |
| 12 | 58·0 | 0·6 | 5·5 | 35·9 |
| 24 | 36·8 | 0·2 | 3·7 | 59·3 |
| 48 | 3·9 | 0·1 | 4·3 | 91·7 |
| Control† | 92·9 | 0·3 | 0·8 | 6·0 |

* 1000 cells scored per smear. Each value represents the mean of five separate experiments.
† Control percentages represent the means of counts at 0 hr and 48 hr.

into discrete membrane-bounded cellular fragments of variable size containing some apparently normal organelles (fig. 4). In this example the nuclear membrane still appears to be intact and there is no evidence of pyknosis.

By contrast, the second type of change, whose incidence was increased from 1 to 48 hr after treatment with a peak at 4 hr (table), involved " rounding up " of the cells. These cells showed loss of protrusions and long slender surface processes and this was accompanied by marked nuclear pyknosis and fragmentation (figs. 5 and 6). In smears, the cytoplasm became intensely staining and vacuoles appeared to congregate together within the cytoplasm (fig. 5). This was confirmed by TEM (fig. 6) which demonstrated aggregation of cytoplasmic organelles: several mitochondria with cristae were visible. The pyknotic nuclei showed nuclear membrane breakdown (fig. 6).

Whilst these were the earliest morphological changes, from 3 hr onwards after treatment an increasing percentage of cells and fragments began to show

more marked degenerative changes (table), and at 48 hr over 90 per cent. showed features of autolysis (Trump and Ginn). In smears, these cells were recognised by their diminished intensity of nuclear and cytoplasmic staining. Ultrastructural features typical of this late-stage process of cell death included focal dissolution of the plasma membrane and breakdown of cytoplasmic organelles, distended mitochondria with flocculent matrix densities and loss of cristae, and further dissolution of the fragmented nucleus, the nucleoplasm becoming increasingly electron lucent (fig. 7).

About 4 per cent. of the cells appeared to have survived steroid treatment and retained normal morphology 48 hr after treatment (table).

Small numbers of necrotic cells were also seen in control cultures (table). In smears these cells contained pyknotic nuclear fragments, and " blebbing " cells and cellular fragments, with or without nuclear remnants, were also seen. Ultrastructurally, some cells showed dilatation of the endoplasmic reticulum and Golgi apparatus, with indentation of the nucleus and distension of the nuclear envelope. Other ultrastructural changes typical of autolysis were also occasionally observed. These observations suggest that death by both " rounding up " and " blebbing ", followed by further degenerative changes, were also naturally occurring in normal cultures.

### *SEM observations on lethal glucocorticoid response*

Control $BLA_1$ cells were basically round with a " ruffled " surface, and occasionally processes were seen adhering to the substrate (fig. 8). After addition of 500 $\mu$g/ml MPS there was an increase in the number of cells showing " blebbing " on an otherwise smooth cell surface (fig. 9), and this reached a peak 2–3 hr after treatment. Cells were examined up to 12 hr after treatment, over which period there was a gradual increase in the number of small, subcellular particles in the samples, and of cells with an irregular surface morphology (fig. 10), which presumably were " rounding up " cells. A gradual accumulation of enlarged cells and cell fragments were also seen and these were undoubtedly autolytic.

## Discussion

The $BLA_1$ cell line studied had morphological features typical of human lymphoblastoid cells (Nilsson, 1971; Nilsson and Pontén, 1975).

In treated cultures, many of the morphological manifestations of the lethal response to 500 $\mu$g/ml MPS were consistent with apoptosis (Kerr *et al.*, 1972). The features are also similar to " popcorn " and " ballooning " processes of cell death described in Hela cells after treatment with lymphotoxin (Russell, Rosenau and Lee, 1972).

In our system, from 1 hr onwards after treatment with MPS, cells died by either " blebbing " or " rounding up ". " Blebbing " cells showed contortion of the cytoplasm and nucleus resulting in the formation of discrete membrane-bounded fragments of variable size containing some apparently normal organelles; in some cases nuclear pyknosis preceded cellular and nuclear

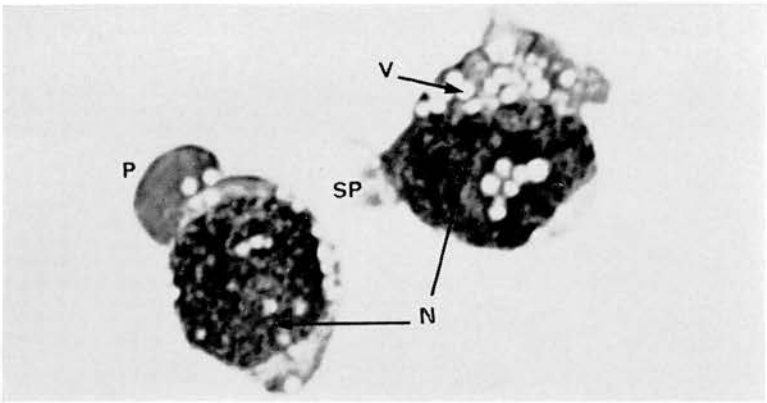


FIG. 1.—Control $BLA_1$ cells. The cell surface shows cytoplasmic protrusions (P) with numerous long slender surface processes (SP), and the cytoplasm contains many clear vacuoles (V). The cells have a high nuclear to cytoplasmic ratio, with the nuclei (N) showing diffusely dispersed chromatin. Giemsa (G). ×1500.

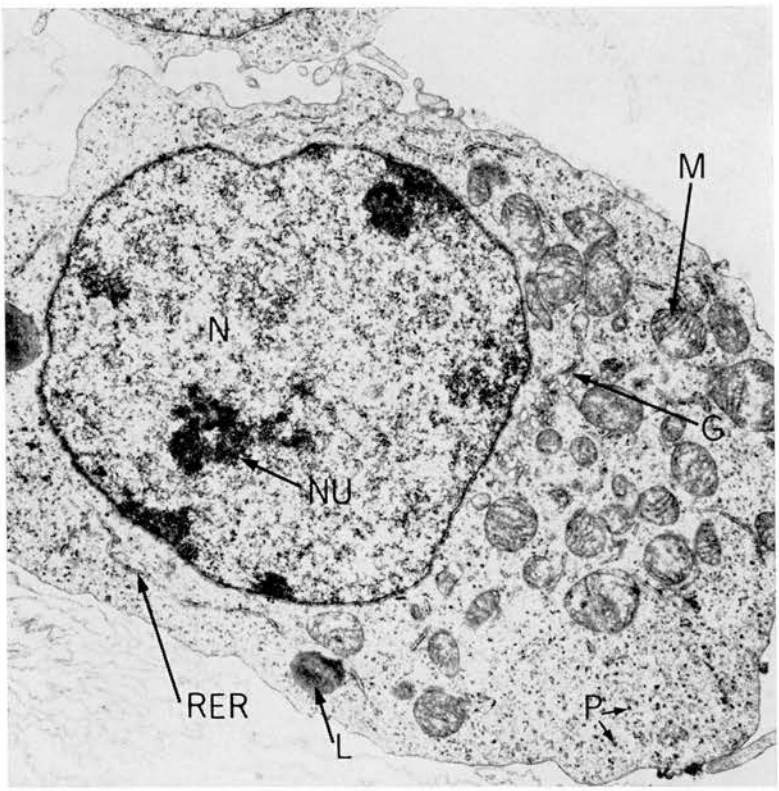


FIG. 2.—Transmission electron micrograph (TEM) of control $BLA_1$ cell. The cytoplasm is rich in polysomes (P) with scanty profiles of rough endoplasmic reticulum (RER), mitochondria with plate-like cristae (M), Golgi apparatus (G) and lipid droplets (L). The nucleus (N) shows diffusely dispersed chromatin with a prominent nucleolus (NU). Some scanty aggregates of heterochromatin are seen along the nuclear membrane. Uranyl acetate and lead citrate (UALC). ×8000.

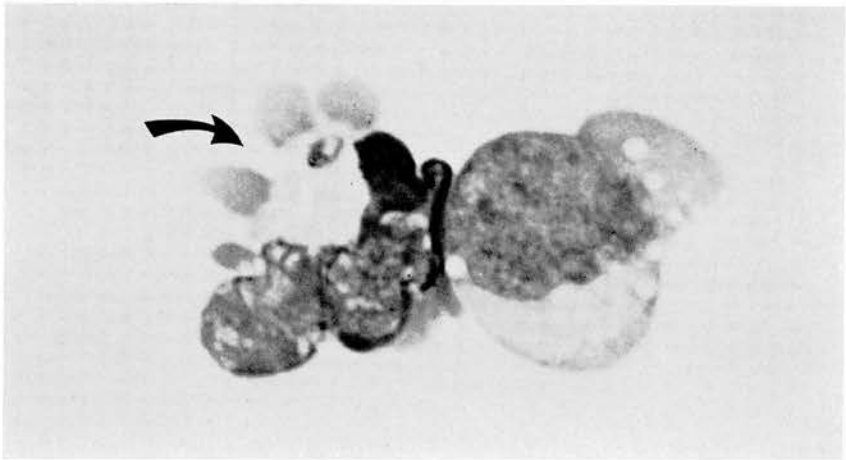

FIG. 3.—$BLA_1$ cells 2 hr after treatment with 500 $\mu$g/ml MPS. The cell (arrowed) shows marked cytoplasmic and nuclear blebbing. The nucleus shows pyknosis and fragmentation. G. ×4000.

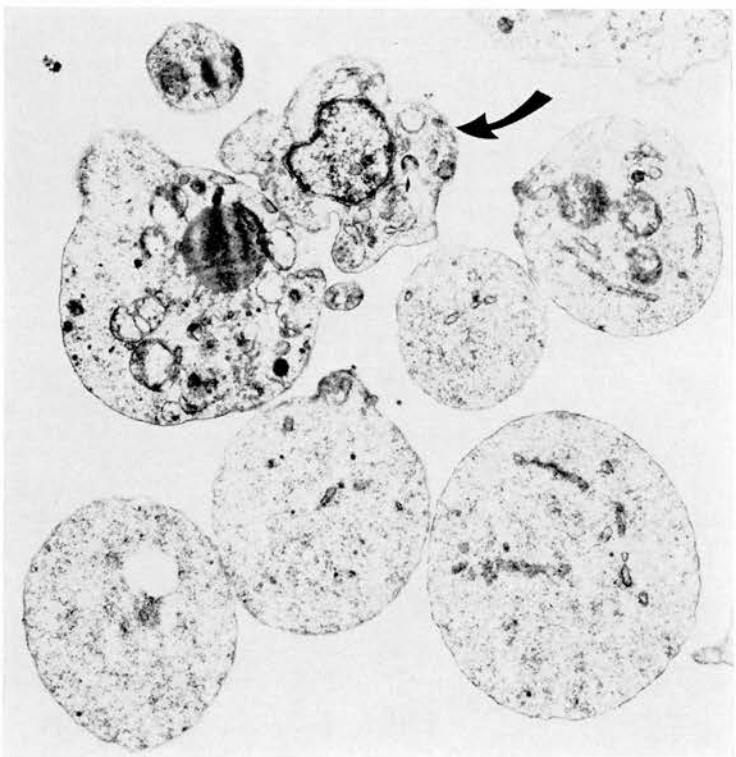

FIG. 4.—TEM of $BLA_1$ cell 1 hr after treatment with 500 $\mu$g/ml MPS. The cell is represented by a cluster of membrane-bounded fragments, most of which contain structurally recognisable organelles and one of which contains nuclear material (arrowed). UALC. ×8800.

GLUCOCORTICOID-INDUCED CELL DEATH

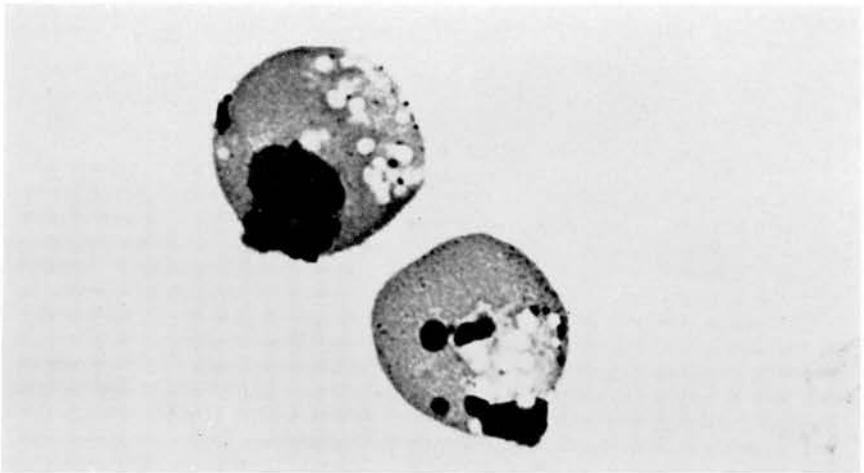

FIG. 5.—$BLA_1$ cells 4 hr after treatment with 500 $\mu$g/ml MPS. The cells show rounding up with loss of surface protrusions and slender surface processes. The cytoplasm contains aggregated vacuoles. The nuclei show pyknosis and fragmentation. G. ×4000.

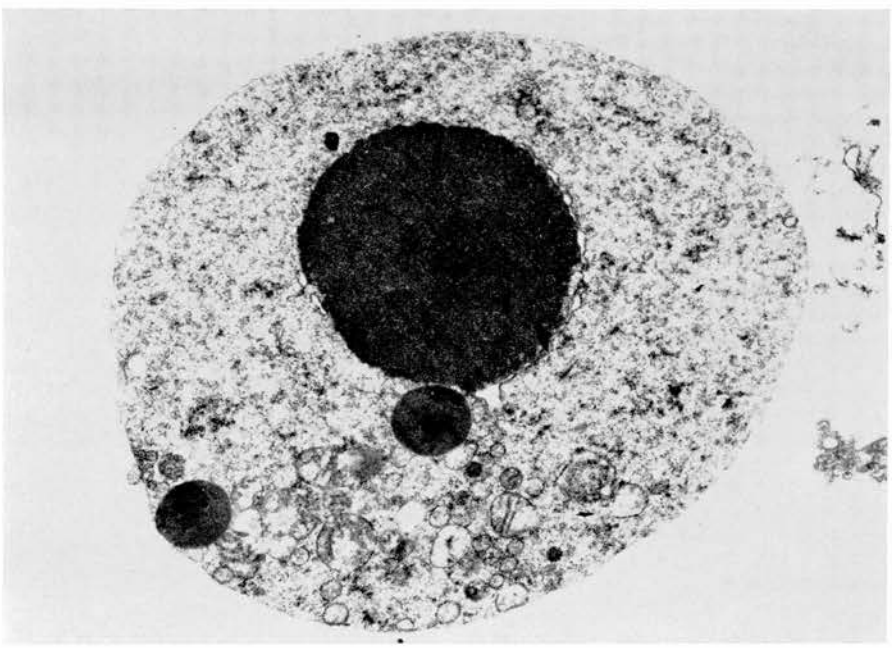

FIG. 6.—TEM of $BLA_1$ cell 6 hr after treatment with 500 $\mu$g/ml MPS. The cell shows rounding up with loss of surface protrusions and slender surface processes and cytoplasmic organelles are aggregated together. The nucleus shows pyknosis with nuclear membrane breakdown. UALC. ×9300.

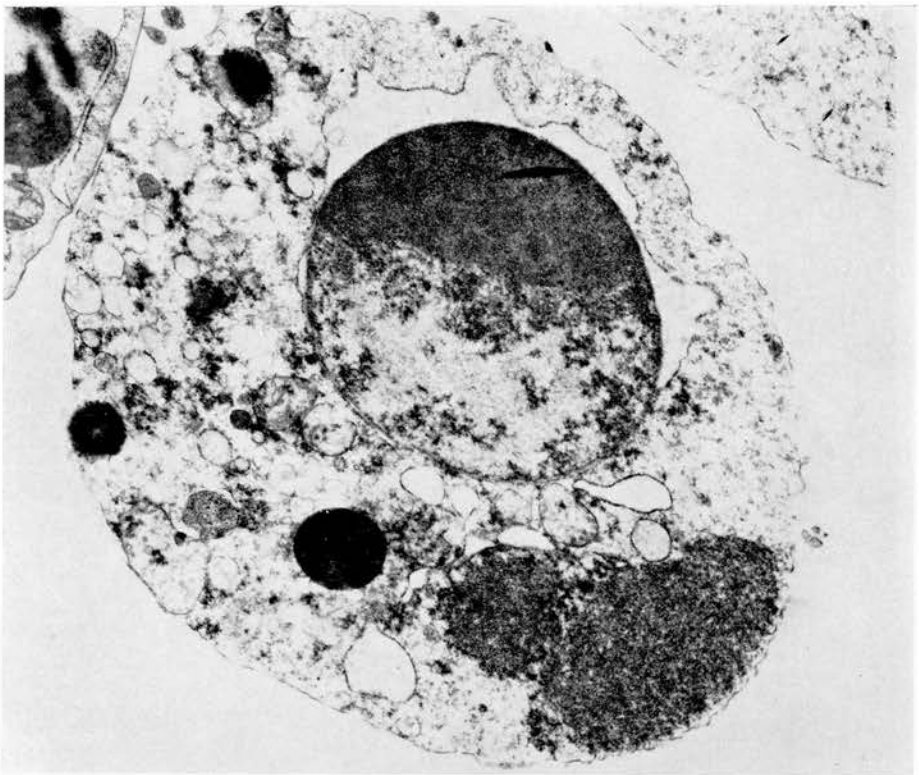

FIG. 7.—TEM of $BLA_1$ cell 4 hr after treatment with 500 $\mu$g/ml MPS. The cell shows marked degenerative changes with focal dissolution of the plasma membrane and loss of structure of cytoplasmic organelles. The pyknotic and fragmented nucleus is becoming electron lucent. UALC. ×13,800.

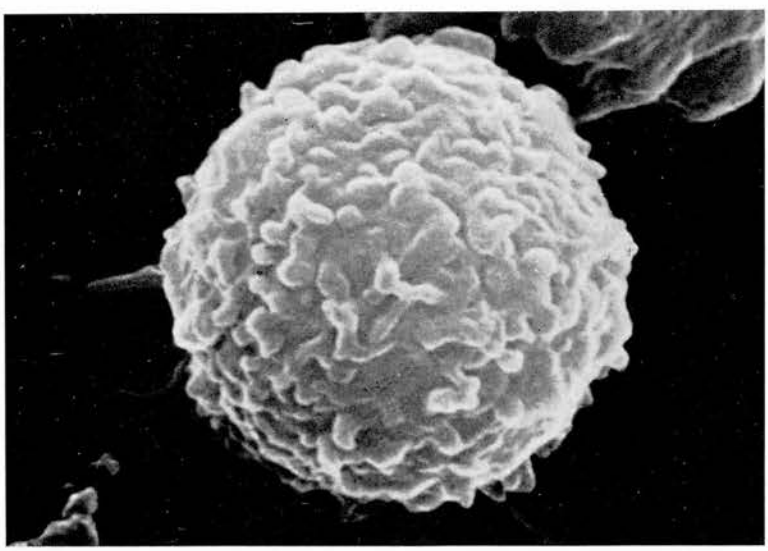

FIG. 8.—Scanning electron micrograph (SEM) of control $BLA_1$ cell. The cell shows a "ruffled" surface with processes adhering to the substrate. ×10,400.

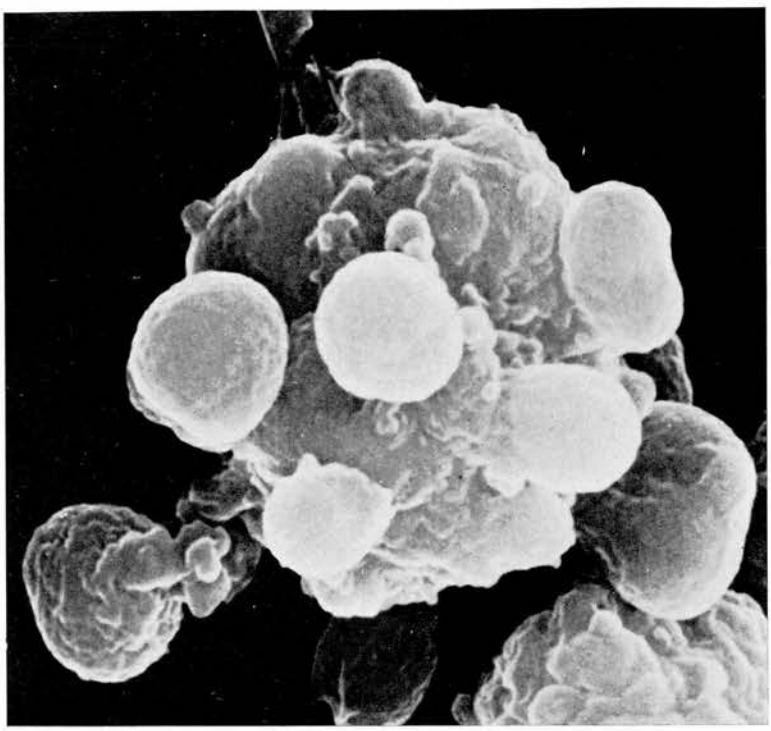

FIG. 9.—SEM of $BLA_1$ cell 4 hr after treatment with 500 $\mu$g/ml MPS. The cell shows " blebbing " on an otherwise smooth cell surface. ×9000.

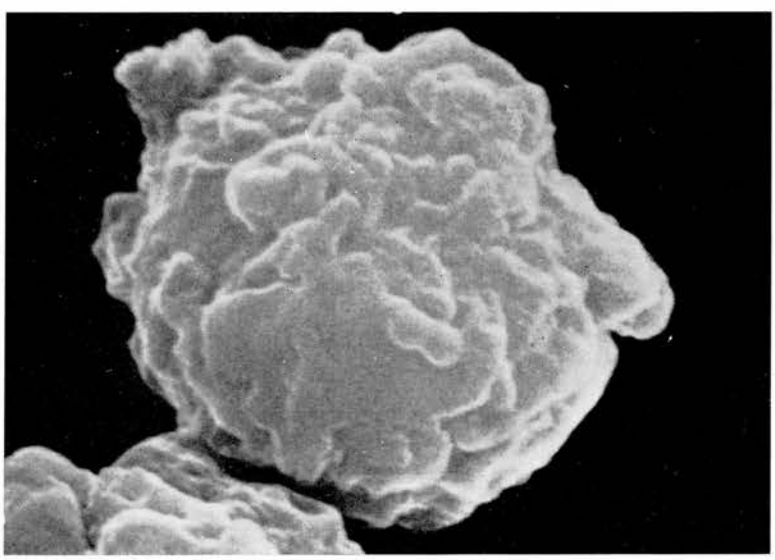

FIG. 10.—SEM of $BLA_1$ cell 4 hr after treatment with 500 $\mu$g/ml MPS. The cell shows an irregular surface. ×10,400.

fragmentation. All these changes occur in apoptosis *in vivo* (Kerr *et al.*). The " rounding up " cells also showed morphological changes typical of apoptosis: loss of cytoplasmic protrusions and long slender surface processes, condensation and preservation of structure of cytoplasmic organelles, and nuclear pyknosis and fragmentation.

Both the " blebbing " and " rounding up " forms of cell death appear to have certain features in common, but from these studies it is not possible to say whether they are two distinct early changes or whether one form precedes the other (see table). We cannot claim that " rounding up " precedes " blebbing " which is the established sequence of events in apoptosis *in vivo* (Kerr *et al.*). However, it seems likely that the response of cells in suspension culture *in vitro* will differ in some respects from that of cells " organised " *in vivo*.

From 3 hr onwards after treatment, increasing numbers of dying cells and fragments showed further degradative changes typical of coagulative necrosis *in vivo* (Trump and Ericsson, 1965; Trump and Arstila, 1971) and of autolysis *in vitro* (Trump and Ginn, 1969) or post-mortem autolysis. Such degenerative changes are found in apoptosis *in vivo* after apoptotic bodies are phagocytosed by surrounding viable cells. However, it seems reasonable to suggest that in an in-vitro system, in the absence of phagocytes, " autolysis " would be expected to occur in apoptotic bodies. We suggest that the morphological changes demonstrated in our system are consistent with apoptosis followed by autolysis.

Certain in-vivo studies also support such a possibility: in mouse ascites tumour cells treated with various non-steroidal cancer chemotherapeutic agents (Searle *et al.*, 1975), many tumour cells which initially undergo apoptosis, ultimately exhibit the features of coagulative necrosis while lying free in ascitic fluid, where apparently there are few active phagocytes. The same sequence of changes has also been noted in apoptotic cells shed into the glandular lumina of the involuting rat prostate in castration-induced atrophy (Kerr and Searle, 1973).

The morphology of spontaneous cell death in control cultures *in vitro* has received scant attention and it is of interest that we found precisely the same sequence of changes—albeit affecting only a small proportion of cells (see table) —as described after treatment with methylprednisolone.

Since human lymphoblastoid cells treated *in vitro* with glucocorticoids show a sequence of morphological changes closely similar to apoptosis *in vivo* we believe this provides a suitable system for studying the early biochemical events and intracellular control mechanisms involved in apoptosis.

## Summary

Morphological aspects of cell death associated with a cytolethal concentration of methylprednisolone sodium succinate (500 $\mu$g/ml) on the $BLA_1$ lymphoblastoid cell line were studied over a 48-hr incubation period by light, transmission and scanning electron microscopy.

Studies revealed two distinctive morphological changes induced by the steroid from 1 hr onwards after treatment. One showed contortion and

" blebbing " of the cytoplasm and nucleus accompanied or followed by nuclear pyknosis, resulting in the formation of membrane-bounded bodies containing apparently normal cytoplasmic organelles with or without nuclear fragments. The other showed " rounding up " of the cell with loss of cytoplasmic protrusions and long slender surface processes, aggregation of well-preserved cytoplasmic organelles, accompanied by nuclear pyknosis and fragmentation. In both cases many of the features are typical of apoptosis. The subsequent degeneration of cells and fragments not unexpectedly resembled in-vitro autolysis.

This in-vitro system is suitable for studying the early biochemical events and intracellular control mechanisms of apoptosis.

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## REFERENCES

BIRD, C. C., ROBERTSON, ALISON M. G., READ, JUDY, AND CURRIE, A. R. 1977. Cytolethal effects of glucocorticoids in human lymphoblastoid cell lines. *J. Path.*, **123**, 145.

BIRD, C. C., WADDELL, A. W., ROBERTSON, ALISON M. G., CURRIE, A. R., STEEL, C. M., AND EVANS, J. 1975. Cytoplasmic receptor levels and glucocorticoid response in human lymphoblastoid cell lines. *Br. J. Cancer*, **32**, 700.

BURTON, A. F., STORR, JUDY M., AND DUNN, W. L. 1967. Cytolytic action of corticosteroids on thymus and lymphoma cells *in vitro*. *Canad. J. Biochem.*, **45**, 289.

DEVITA, V. T. 1973. Combined drug treatment of Hodgkin's disease: remission induction, remission duration, and survival; appraisal. *Natn Cancer Inst. Monogr.*, **36**, 373.

DOUGHERTY, T. F., AND WHITE, A. 1945. Functional alterations in lymphoid tissue induced by adrenal cortical secretion. *Amer. J. Anat.*, **77**, 81.

HAWKINS, H. K., ERICSSON, J. L. E., BIBERFELD, P., AND TRUMP, B. F. 1972. Lysosome and phagosome stability in lethal cell injury. Morphologic tracer studies in cell injury due to inhibition of energy metabolism, immune cytolysis and photosensitization. *Amer. J. Path.*, **68**, 255.

KERR, J. F. R., AND SEARLE, J. 1973. Deletion of cells by apoptosis during castration-induced involution of the rat prostate. *Virchows Arch. Abt. B Zellpath.*, **13**, 87.

KERR, J. F. R., WYLLIE, A. H., AND CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, **26**, 239.

LAIRD, ANNA K. 1969. Dynamics of growth in tumors and in normal organisms. *Natn Cancer Inst. Monogr.*, **30**, 15.

MCDOWELL, ELIZABETH M. 1972. Light and electron-microscope studies of the rat kidney after administration of inhibitors of the citric acid cycle *in vivo*: changes in the proximal convoluted tubule during fluorocitrate poisoning. *J. Path.*, **108**, 303.

MCLEAN, A. E. M., MCLEAN, ELIZABETH, AND JUDAH, J. D. 1965. Cellular necrosis in the liver induced and modified by drugs. *Int. Rev. Exp. Path.*, **4**, 127.

MAJNO, G. 1964. Interactions between dead cells and living tissue. *In* Ciba Foundation symposium on cellular injury, edited by A. V. S. de Reuch and J. Knight, Churchill, *London*, p. 87.

NILSSON, K. 1971. Characteristics of established myeloma and lymphoblastoid cell lines derived from an E myeloma patient: a comparative study. *Int. J. Cancer*, **7**, 380.

NILSSON, K., AND PONTÉN, J. 1975. Classification and biological nature of established human hematopoietic cell lines. *Int. J. Cancer*, **15**, 321.

RUSSELL, S. W., ROSENAU, W., AND LEE, J. C. 1972. Cytolysis induced by human lymphotoxin. Cinemicrographic and electron microscopic observations. *Amer. J. Path.*, **69**, 103.

SALADINO, A. J., AND TRUMP, B. F. 1968. Ion movements in cell injury. Effects of inhibition of respiration and glycolysis on the ultrastructure and function of the epithelial cells of the toad bladder. *Amer. J. Path.*, **52**, 737.

SCHEIN, P. S., CHABNER, B. A., CANELLOS, G. P., YOUNG, R. C., BERARD, C., AND DEVITA, V. T. 1975. Results of combination of chemotherapy of non-Hodgkin's lymphoma. *Br. J. Cancer*, **31**, Suppl. II, 465.

SEARLE, J., LAWSON, T. A., ABBOTT, P. J., HARMON, B., AND KERR, J. F. R. 1975. An electron microscope study of the mode of cell death induced by cancer-chemotherapeutic agents in populations of proliferating normal and neoplastic cells. *J. Path.*, **116**, 129.

SIMONE, J. 1974. Acute lymphocytic leukaemia in childhood. *Semin. Haematol.*, **11**, 25.

STEEL, G. G. 1967. Cell loss as a factor in the growth rate of human tumours. *Europ. J. Cancer*, **3**, 381.

TRUMP, B. F., AND ARSTILA, A. U. 1971. Cell injury and cell death. *In* Principles of pathobiology, edited by M. F. La Via and R. B. Hill, Jr, Oxford University Press, *New York*, *London*, and *Toronto*, p. 9.

TRUMP, B. F., AND ERICSSON, J. L. E. 1965. Some ultrastructural and biochemical consequences of cell injury. *In* The inflammatory process, edited by B. W. Zweifach, L. Grant and R. T. McCluskey, Academic Press, *New York* and *London*, p. 35.

TRUMP, B. F., AND GINN, F. L. 1969. The pathogenesis of subcellular reaction to lethal injury. *In* Methods and achievements in experimental pathology, edited by E. Bajusz and G. Jasmin, Karger, *Basel*, vol. 4, p. 1.

WADDELL, A. W., BIRD, C. C., AND CURRIE, A. R. 1977. Effect of methylprednisolone on the nucleoside metabolism of a human lymphoblastoid cell line. *Br. J. Cancer*, **36**, 187.

WHITFIELD, J. F., PERRIS, A. D., AND YOUDALE, T. 1968. Destruction of the nuclear morphology of thymic lymphocytes by the corticosteroid cortisol. *Expl. Cell Res.*, **52**, 349.

WYLLIE, A. H., KERR, J. F. R., MACASKILL, I. A. M., AND CURRIE, A. R. 1973. Adrenocortical cell deletion: the role of ACTH. *J. Path.*, **111**, 85.